BIODEGRADATION OF BISPHENOL A AND IBUPROFEN BY AMMONIA OXIDIZING BACTERIA

A Thesis

by

NETHRA T. SUBRAMANYA

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements of the degree of

MASTER OF SCIENCE

May 2007

Major Subject: Civil Engineering

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Approved by:

Chair of Committee,	Kung-Hui Chu
Committee Members,	Robin Autenrieth
	Kirby Donnelly
Head of Department,	David Rosowsky

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ABSTRACT

Biodegradation of Bisphenol A and Ibuprofen
by Ammonia Oxidizing Bacteria. (May 2007)
Nethra T. Subramanya, B.E, Visvesvaraya Technological University, India
Chair of Advisory Committee: Dr. Kung-Hui Chu

Bisphenol A (BPA) is a compound that is commonly used in the manufacture of epoxy resins and plastics. Because of large scale production and widespread usages, BPA is released into the atmosphere through air, land, and water. BPA is weakly estrogenic in animals and has acute aquatic toxicity even at low concentrations of 1-10μg/L. Ibuprofen is a widely used analgesic and antipyretic. Ibuprofen and its metabolites are mainly released into the environment by human urinary excretion. Ibuprofen has been detected at low concentrations in surface and waste waters. The environmental and health effects at such concentrations are unclear.

The high removal of BPA and ibuprofen in the wastewater treatment plants (WWTPs), suggest that biodegradation might be responsible for the removal of these compounds. Several bacterial strains, isolated from waste water, are known to degrade BPA and ibuprofen. No studies, however, have reported using ammonia oxidizing bacteria for this purpose. Ammonia oxidizing bacteria (AOB) are an important group of microorganisms in nitrifying activated sludge of WWTPs. AOB are known to express ammonia monooxygenase (AMO) to degrade many different aromatic and aliphatic organics via cometobolic degradation (non beneficial mechanism). *Nitrosomonas europaea* is a widely studied AOB found to degrade synthetic estrogen by a study.

This study aims to characterize the biodegradation of BPA and ibuprofen by AOB. The biodegradation ability of *N.europaea* with respect to BPA and ibuprofen was examined. Experiments were conducted in the presence/absence of the AMO inhibitor (allylthiourea), an external reducing energy source (sodium formate) and different

primary substrate (ammonia) concentrations. The second part of the study comprises of biodegradation tests on BPA and ibuprofen using activated sludge from two WWTPs, one with one-sludge activated sludge system and the other one with two-sludge nitrification system.

From the experiments conducted BPA at a concentration of 1.6 mg/L was degraded to 0.12 mg/L by *N.europaea*. BPA at concentrations of 1.0 mg/L and 0.75 mg/L was completely degraded by the cells. Resting cells of *N.europaea* were, however, unable to degrade BPA. Also ibuprofen of two concentrations, 0.42 mg/L and 0.8 mg/L, were not degraded by the culture.

BPA at a concentration of 1 mg/L was degraded to 0.2 mg/L and ibuprofen at 0.5 mg/L was completely degraded by the activated sludge from the combined reactor. The activated sludge from the nitrification tank degraded BPA of concentration 1 mg/L and ibuprofen of concentration 0.5 mg/L completely.

Hence, it can be summarized that Bisphenol A was degraded by *N.europaea* and also by the activated sludge obtained from the WWTPs. Ibuprofen was found incapable of inhibiting ammonia oxidizing bacteria in the case of the pure culture while it was successfully degraded by the mixed culture.

Dedicated

to

Amma, Appa, Ganesha

and

Narahari

ACKNOWLEDGMENTS

Performing research is no simple task. It, possibly, can get harder if you do not have the support and encouragement of many people. In this page, I would like to gratefully acknowledge these people without whose support and guidance, I would have woefully fallen short of my achievement.

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CHAPTER I
INTRODUCTION

Bisphenol A (BPA) is an intermediate product in the manufacture of compounds like plastics, epoxy resins and polycarbonate resins [1]. BPA was first discovered in the year 1905 and since then its commercial popularity has increased gradually with time. Presently BPA is being used in a number of products like adhesives, building materials, powder paints, inner coating of cans, automotive lenses, compact discs, thermal paper, for encapsulation of electrical and electronic parts and so on [49]. Production of BPA in 2001 alone was approximately equal to 2.5 million tons which increased to 2.8 million tons in the year 2002 [50, 1].

BPA is detected in surface waters at a concentration up to 0.4 μg/L [3]. BPA is released into the environment during manufacturing processes like heating, handling and transportation. Accidental spills and leakages are also a major source. BPA is known to leach specifically from plastic products, most prominent being poly vinyl chloride [7]. BPA causes acute toxicity to aquatic organisms between concentrations of 1-10 μg/L [2]. BPA is also an endocrine disrupter and studies have confirmed its estrogenicity and effects on the behavior of mice [8, 29, 43]. Removal of BPA by wastewater treatment plants (WWTPs) has been reported to be greater than 90% [11, 49]. Several bacterial strains isolated from wastewater can use BPA as a growth substrate [20, 22, 23, 32, 43].

Ibuprofen is a popular chiral pharmaceutical drug classified as a non-steroidal anti-inflammatory drug (NSAID) [42]. Ibuprofen is used as an analgesic, antipyretic and against anti-inflammatory conditions. Ibuprofen is one of the many pharmaceutical compounds that are increasingly being detected in influents to WWTPs. Ibuprofen is persistent and mobile in the aquatic environment because of its physical and chemical properties. Ibuprofen is used extensively as a pharmaceutical drug since it can be obtained without a prescription [42]. Ibuprofen's concentration in surface waters has been reported to be between 2-8 ng/L in most lakes, with some German rivers containing up to 139 ng/L [42].

This thesis follows the style of Journal of Applied and Environmental Microbiology (AEM).

There is growing concern regarding the increasing presence of ibuprofen and its metabolites in WWTPs and surface waters. Ibuprofen is known to effect the growth of certain species of aquatic plants, *Synechocystis* sp. and *Lemna minor* [39]. However information on its environmental and health effects at low concentrations is limited and unclear.

The WWTPs are capable of removing approximately 95% of ibuprofen and its metabolites [42]. Studies conducted in the laboratory by incubating WWTP samples indicate the biodegradation of ibuprofen [42]. However there are a limited number of studies conducted on the biodegradation of ibuprofen. One study reported ibuprofen degradation by a bacterial strain belonging to the species *Nocardia* [5]. Another study isolated a *Sphingomonas* sp. strain from wastewater as being capable of degrading ibuprofen [37]. In both studies ibuprofen was used as a growth substrate by the bacterial strains.

The biodegradation of both BPA and ibuprofen has been described in earlier research [5, 20, 22, 23, 32, 37, 43]. The present study is aimed at examining the degradation possibilities of both these compounds by AOB. Ammonia oxidizing bacteria (AOB) are important microorganisms found in the nitrifying activated sludge of WWTP's known to degrade several compounds [25]. Studies have reported a wide substrate range for these bacteria which includes both aromatic and aliphatic compounds [5]. *Nitrosomonas europaea,* a highly researched AOB is known to degrade synthetic estrogens as per a recent study [46]. However no study has focused on utilizing the AOB and *N.europaea* for degrading BPA and ibuprofen.

This study is based on the hypothesis that ammonia oxidizing bacteria might play an important role in removing BPA and ibuprofen via cometabolism during biological wastewater treatment.

1.1 GOAL AND OBJECTIVES

The goal of this study is to understand the biodegradation of both BPA and ibuprofen by AOB. The main objectives of the study are:

1. To characterize the co-metabolic degradation of BPA and ibuprofen by an ammonia oxidizing bacterium, *Nitrosomonas europaea.*
2. To study the biodegradation of BPA and ibuprofen by ammonia oxidizing bacteria from the nitrifying activated sludge collected from two waste water treatment plants.

The following chapters cover more information on the two compounds, the ammonia oxidizing bacteria, the experimental materials and methods, results and discussion.

CHAPTER II

BACKGROUND

2.1 PHYSICAL AND CHEMICAL PROPERTIES

2.1.1 BPA. BPA is an aromatic compound that is synthetically manufactured and used as a chemical building block in the manufacture of several products [1]. These products are light-weight, resistant to high temperatures, tough and adhesive in nature; making BPA a highly useful industrial compound [1].

The structure of BPA consists of two phenol groups with two methyl groups connecting them (Table 1) [49]. BPA occurs as a solid at room temperature and standard pressure in the form of prills, flakes and crystals [49]. BPA has a low volatility (lesser than water) as compounds with Henry's constant < 1.0E-7 atm-m^3/mol (Table 1) are considered to be less volatile [15, 49].

BPA has greater solubility at alkaline pH [15, 49]. It is considered to be hydrophobic and is known to partition to soils and sediments [7]. However some part of it is present dissolved in the aqueous phase based on the concentration [3]. BPA is not known to ionize in the environment with pH values around 7.0 since it has a pKa value between 9.59 and 11.30 [7]. But BPA can ionize under alkaline conditions that are normally prevalent in the industries [7].

2.1.2 Ibuprofen. Ibuprofen is an aromatic compound that is manufactured to be used as a drug in the pharmaceutical industry. Ibuprofen was first discovered in Nottingham, England by Dr. Stuart Adams, John Nicholson and Collin Burrows [15]. Ibuprofen is an ingredient in some common drugs available in the market like Deep Relief, Nurofen, Nurofen gel and Nurofen Plus used to treat common ailments like fever, pain and rheumatic disorders [15, 42]. Ibuprofen specifically is used to treat pains that occur due to menstruation, headaches, inflammatory conditions like rheumatoid arthritis and some common ailments like fever [16]. It is prescribed in high dosages of the order of 600-1200 mg/d [42]. Around 70-80% of the dosage prescribed is released into the environment through excretion. Along with Ibuprofen, four other metabolites have also been detected in the environment [42].

Ibuprofen has a propionic acid group attached to its structure (Table 1) [16]. Ibuprofen has a molecular weight of 206.29 g/mol with a CAS number 15687-27-1 [16]. Ibuprofen occurs as a white crystalline powder at room temperature and standard pressure [16]. Ibuprofen is reported to be practically insoluble in water [16]. Ibuprofen has a melting point between 75°C-78°C and is a relatively stable compound at room temperature and standard pressure [16].

Ibuprofen has two enantiomers namely the *S*-ibuprofen and *R*-ibuprofen as shown in Table 1 [42]. During the metabolism in the body *R*-ibuprofen is converted into the pharmacologically active *S*-enantiomer [42].

TABLE 1. Structure and chemical properties of BPA and ibuprofen.

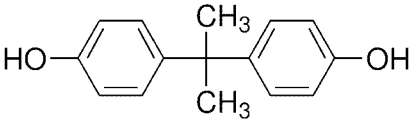
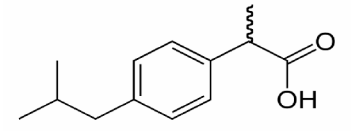
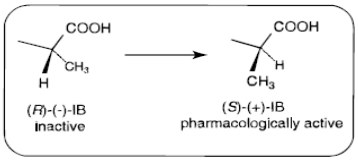
Property	**BPA**	**Ibuprofen**
Figure [59, 16]		 **Structure of ibuprofen enantiomers** 
Chemical formula [49, 16]	$C_{15}H_{16}O_2$	$(CH_3)_2CHCH_2C_6H_4CH(CH_3)COOH$
Molecular weight [49, 16]	228 g/mol	206.29 g/mol

TABLE 1. Continued.

CAS Number [49, 16]	80-05-7	15687-27-1
Solubility [49, 16]	120-300mg/L	Practically insoluble
Melting point [15, 16]	150°C-155°C	75°C-78°C
Boiling point [15]	220°C at 4mm of Hg	-
Henry's constant [15]	1.0E-10 atm-m^3/mol	-
Koc [15]	314-1584 at water solubility of 120 mg/L	-

2.2 ENVIRONMENTAL SOURCES, OCCURRENCE AND FATE

2.2.1 BPA. BPA enters the environment through several pathways. BPA is released into the environment during the manufacturing process (particularly during heating, handling, transporting the material and accidental spills) [49]. BPA is known to have a low vapor pressure of 5.3x 10^{-6} Pa. During the manufacturing processes, it is heated to high temperatures at which it is released in the vapor form to the atmosphere [7]. BPA being moderately soluble in water enters into the environment by the wastewater generated during the manufacturing process. The wash water used is another major source [7]. Despite the treatment of these waste waters, complete removal of BPA is not ensured. Due to BPA's low air water partition coefficient of 10^{-9}, the BPA in water does not evaporate [7].

BPA also enters the environment by the disposal of the products in which it is used like the lining of cans or from plastic products [49]. BPA enters the soil from the leachates of waste landfills which have a BPA concentration of 1.3-17,200 μg/l [59].

According to the US Toxics Release Inventory (TRI), 85,300 kg is released through fugitive and stack emissions, about 3500 kg is directly released to water, 1100 kg is released by effluents from WWTPs (> 90% removal) and 10,000 kg to water from thermofax paper, landfills, leakages from linings of pipes, products that are not landfilled and dust formation was reported by Cousins, Staples, Klecka and Mackay in 2002 [7].

Approximately around 65% of the produced BPA is used in the manufacture of polycarbonate resins, 28% in epoxy resins and the remaining 7% is used in other miscellaneous products [7]. BPA emissions from various point sources were detected ranging from 2.1 μg/L to 41 μg/L according to a study (Table 2) [11]. The samples were taken across ten sampling sites with four times a day, five days a week within a period of five months to overcome fluctuations [11].

TABLE 2. Average emissions of BPA from various point sources. [11]

Sample spots	**Mean concentration (μg/L)**	**Minimum value**	**Maximum value**
Metal/wood industry	17	2.6	35
Chemical industry	18	2.5	50
Hospital	non detectable	non detectable	1.0
Paper production	41	28	72
Cloth washing company	5.2	1.0	8.9
Food industry	2.1	non detectable	3.8
Household areas I	2.2	non detectable	2.6
Household areas II	2.5	non detectable	5.8
Influent	21	10	37
Effluent	1.5	non detectable	2.5

2.2.2 Ibuprofen. Ibuprofen is produced on the order of several kilotons every year all over the world as indicated in Table 3 [13, 26, 48, 53, 54, 55]. Ibuprofen has the distinction of being the third most popular drug by usage and sales [41].

TABLE 3. Ibuprofen usage across countries. [13, 26, 48, 53, 54, 55].

Country/ Year	Quantity (ton/yr)
United Kingdom/ 2000	162
Denmark/ 1997	34
Deutschland	105-180
Australia	14
United States	Not available

As ibuprofen is used as a drug, it is partly or fully metabolized in the body [12]. During the metabolism, the parent compound is normally converted into metabolites that could prove to be more or less harmful than the parent compound [12]. Urine tested from a patient who had been administered with ibuprofen showed the presence of metabolites like methyl esters of ibuprofen, carboxy- IB (major), hydroxyl- IB and carboxy-hydratropic acid (carboxy- HA) [42]. Usually the metabolites are more water soluble than the parent compound [12]. Both the parent compound and the metabolites are released into the environment through urine [12].

Ibuprofen is prescribed in high dosages of 600-1200 mg/d and it can also be bought without a prescription [42]. Around 70-80% of this therapeutic dose enters the environment due to excretion [42]. Ibuprofen can also be released into the environment due to the direct disposal of outdated or excess medication [12]. Ibuprofen was detected up to a concentration of 3µg/ L in the influents to WWTPs. However in surface waters they can be detected at a concentration of 8 ng/L [42].

A study conducted on the water systems and rivers in Italy reported the presence of ibuprofen in river water, river sediments and drinking water [61]. Ibuprofen was

detected in a wide cncentration range of 1.0 ng/L to 92.4 ng/L in the three major rivers [61]. In one of the river sediments ibuprofen was detected up to 220 ng/kg [61]. However it was not detectable in the drinking water systems of any of the major cities investigated [61].

2.3 TOXICITY

2.3.1 Toxicity of BPA to ecosystem. BPA is toxic to both freshwater and saltwater species [2]. The following Table 4 summarizes BPA toxicity to freshwater and saltwater species.

TABLE 4. Aquatic toxicity to freshwater and saltwater species. [2]

Freshwater species	**Toxicity**
Selenastrum capricornutum	96 hour EC_{50} based on cell count was 2.7 mg/L, based on cell volume 3.1 mg/L.
Daphnia magna	48 hour EC_{50}, 10 mg/L
Pimephales promelas	96 hour LC_{50} in static test 4.7 mg/L and in flow through test 4.6 mg/L
Saltwater species	**Toxicity**
Skeletonema costatum	96 hour EC_{50} based on cell count 1 mg/L and based on chlorophyll-a content 1.8 mg/L.
Mysidopsis bahia	96 hour LC_{50}, 1.1mg/L
Menidia menidia	96 hour LC_{50}, 9.4 mg/L

2.3.2 Toxicity of BPA to animals. One study confirms the estrogenicity of BPA due to which it binds to estrogen receptors in rat uterus [30]. According to Yoshihara et al, BPA of 0.1 mM (or 22.8 mg/L) concentration increased the estrogenicity in rats by two to five

folds [34]. BPA is reported to increase the height of the luminal epithelial cells in the uterus and also increase the vaginal openings in female mice [34].

BPA also induces progesterone receptors in cancerous mammary cells in human beings [30]. A study on pregnant human mothers exposed to BPA detected significant levels of BPA in the blood and plasma of both the mother and the fetus [44]. BPA levels in the maternal plasma, fetal plasma and placental tissue were found to be 3.1 ng/mL, 2.3 ng/mL and 12.7 ng/g respectively [44]. Exposures of animals to similar concentrations were known to affect some organs of the offspring [30]. BPA is also known to induce depression like behavior in male rats and reduces the sexual differences in rearing behavior [10].

The average environmental concentration of BPA in surface waters is 0.41 μg/L [3]. However the toxicity concentrations reported range from 2.3 mg/L to 22.8 mg/L [10, 30, 34, 44]. A difference of the order of 10^3 exists between the environmental and toxicity concentrations of BPA.

2.3.3 Toxicity of ibuprofen to ecosystem. Ibuprofen is currently detected in low concentrations in the environment with the maximum detected concentration being 8 ng/L [42]. Information on the effects of ibuprofen on the human health and its toxicity is limited. Though in a broader sense, it is known that pharmaceuticals are manufactured to perform a specific biological action [12]. This could either be useful or harmful to other organisms when they are released into the environment [12]. They are also lipophilic and hence are able to travel across different membranes [12].

Ibuprofen is known to affect certain species of aquatic plants like *Synechocystis* and *Lemna* [40]. While ibuprofen stimulates the growth of *Synechocystis* at a concentration of 10 μg/L, it is known to inhibit the growth of *Lemna* at 1 mg/L [39]. Also exposure to ibuprofen resulted in the production of the stress hormone abscisic acid in *Lemna* [39]. Ibuprofen along with certain other chemicals is also known to be harmful to aquatic organisms. A study reported the harmful effect of a mixture of ibuprofen, prozac and ciprofloxacin on certain aquatic plants, planktons and fish at concentrations lower than the human dosage of 600-1200 mg/d [39, 41]. It was observed that the

mixture caused the death of certain species of plankton, sunfish (*Lepomis gibbosus*) and duckweed (*Lemna gibba*) [41].

2.3.4 Toxicity of ibuprofen to animals. Ibuprofen is also known to hinder the cardiovascular protective properties of aspirin (both less than 325 mg/day) consumed by patients with cardiovascular problems [33]. The study was conducted on cardiovascular patients who were administered with aspirin at low dosages [33].

The concentration of ibuprofen of upto 7.8 ng/L was reported for 8 lakes including the North Sea [42]. The toxicity concentrations vary between 10 μg/L to 1 mg/L [39]. Hence a difference of magnitude 10^3 to 10^6 between the environmental and toxicity concentrations is present. This reduces the magnitude of the risks posed by ibuprofen to the ecosystem.

2.4 REMOVAL OF BPA AND IBUPROFEN

2.4.1 BPA. Removal of BPA occurs by both biotic and abiotic processes which are summarized below.

2.4.1.1 Biodegradation:

A. Surface water and WWTP. The biodegradation of BPA has been the focus of several studies as opposed to alternate chemical and physical methods of treatment [9, 32, 47]. BPA is known to be biologically degraded rapidly in surface waters with an average half life of below 5 days [22].

Several aerobic BPA-degrading bacterial strains have been isolated from river water and WWTPs [32, 43, 47]. Two bacterial strains *Pseudomonas* sp. and *Pseudomonas putida* that degraded BPA by more than 90% were isolated from river water [22]. Under aerobic conditions, BPA degradation was > 90% at the end of 7 days compared to a BPA degradation of < 10% after 10 days under anaerobic conditions [22].

A study was conducted on the BPA released by various industries into the Houston ship channel [9]. Water samples from Houston ship channel were spiked with BPA in 1 gallon water columns with air inlets at the bottom and sterililzed water was used as the control [9]. The experiment was conducted in the dark [9]. Degradation in most of the

samples was observed after two days with the concentrations of BPA falling below detectable limits on the 4th day [9]. This reduction was attributed to biodegradation as there was no exposure to UV light or changes in the pH of the samples [9]. Approximately 96-97% of BPA added was degraded within 3 days [9]. Some studies have successfully isolated bacterial strains that are capable of degrading BPA and the various parameters involved in the process. One study reports that an increase in the sludge retention time in WWTPs beyond 40 days does not have any positive effect on the biodegradation of BPA [29]. The ensuing section covers more information on the BPA degrading bacterial strains.

B. Soil. Since BPA has a high octanol-air partition coefficient it tends to sorb on to solid surfaces, vegetation, soils and aerosols [49]. BPA degradation in soil sediments is reported to have a half life of less than 3 days [36]. Metabolites of BPA were also successfully degraded in the soil [36]. Most of the BPA loss was attributed to aerobic biodegradation while a part of the loss was due to incorporation in the soil organic matter [36]. No data regarding the effect of UV on BPA in soil is available.

2.4.1.2 Abiotic removal of BPA. Apart from biodegradation, several abiotic processes have also been used in the treatment of BPA in WWTP's. Chemical degradation of BPA also occurs in the environment by photodegradation [15].

A. Chemical method. Chemical methods have been applied successfully in the pretreatment of BPA in wastewater. One study reported more than 85% removal of BPA by applying pH neutralization [8]. Chemical methods like ozonation and chlorination have also been used for the removal of BPA in drinking water treatment. BPA of concentration 500- 300,000 ng/L was treated with ozonation (1.4 mg/L O_3) and chlorination with sodium hypochlorite (0.5 mg/L) and chlorine dioxide (0.4-0.6 mg/L) [31]. Ozonation followed by chlorination removed BPA below detectable limits [31]. Ozonation combined with sodium hypochlorite treatment also ended in the same results but with an increase in the estrogenic activity [31].

B. Photodegradation. BPA is also degraded by photodegradation in surface waters and in the atmosphere [15]. This process is called photolysis or photooxidation [15]. BPA is

known to absorb UV light of wavelengths greater than 290 nm [15]. This alters the chemical properties of BPA. In the atmosphere BPA is subject hydroxyl radical attacks as well [15]. With respect to photodegradation BPA is known to have a half life of 66 hours to 160 days in water and 0.74 to 7.4 hours in air [15].

C. Physical methods. More than 85 % BPA removal was observed by precipitate filtration and XAD resins [8]. Use of different nanofilters for the removal of BPA reported a removal of 70% to 100% [58]. Other technologies like membrane bioreactors and reverse osmosis met with 98% removal [58]. Treatment by membrane bioreactors accompanied by the use of granulated activated carbon resulted in 99% BPA removal [58].

2.4.2 Ibuprofen. Most of the literature provides data about ibuprofen in water with no available data about ibuprofen's occurrence soil and air. Both ibuprofen and its metabolites in urine were detected in the wastewater influents [42]. Ibuprofen was detected at a concentration of 3.3 μg/L in the influent, while the metabolites hydroxyl-IB and carboxy- IB were found at higher concentrations and hydroxyl-IB was found in lower concentration [42]. Effluent concentrations of ibuprofen, carboxy-IB and hydroxyl-IB were detected at a much lower range 9 to 81 ng/L [42]. This indicated that ibuprofen was efficiently removed by WWTPs.

It was found that ibuprofen and carboxy-IB dominated the raw sludge while hydroxyl-IB was more prominent in the treated waters [38]. In a WWTP that had primary settling, chemical removal of phosphorous, activated sludge and biological nitrogen removal processes, the removal of ibuprofen and carboxy-IB was more than 95% [38]. The removal of hydroxyl-IB was reported to be 60% due to the formation of 40% hydroxyl-IB during the removal process [38]. Additionally disturbances in a WWTP with only primary settling and activated sludge processes resulted in a lower removal (43%) of ibuprofen and almost no removal of hydroxyl-IB [38]. However this did not seem to affect the removal of carboxy-IB [38].

2.4.2.1 Biodegradation. Biodegradation is known to be the main process for removal of ibuprofen from the environment with the formation of polar metabolites in WWTPs [38].

While the biodegradation of ibuprofen has been reported several times in the WWTP's, only two ibuprofen degrading cultures were reported. One study conducted earlier reported the side chain hydroxylation of the compound in biofilm reactors [62]. This was observed to occur under both aerobic and anaerobic conditions resulting in the degradation of ibuprofen with the formation of metabolites [62].

Degradation tests of ibuprofen with activated sludge conducted in the laboratory resulted in a decrease in the concentration after a period of 8 hours [42]. Influent to a WWTP containing ibuprofen and its metabolites were mixed with the activated sludge by air diffusion from the same WWTP in glass bottles [42]. The *S*-enantiomer is degraded at a faster while the *R*-enantiomer is more persistent [42].The study also reported a retention time of more than 6 hours for the efficient degradation of ibuprofen [42]. Tests conducted on fortified lake water also reported similar results with the *S*-enantiomer being degraded faster than the *R*-enantiomer. The degradation occurred both in daylight and during the night [42].

Another laboratory study reports that increased sludge retention time resulted in enhanced degradation of ibuprofen [52]. However there was no such conclusion from the study conducted on the full scale plants [52]. This study also reported the near complete removal of ibuprofen in WWTPs, specifically in the ones that were designed for nitrogen removal or for nitrification [52]. The complete removal was consistent both in the case of full scale plants as well as laboratory experiments [52].

2.4.2.2 Abiotic (physical) processes. A study was conducted to use ozonation, nanofiltration, microfiltration and reverse osmosis to remove pharmaceuticals like ibuprofen from treated wastewater for water recycle [27]. Ibuprofen of concentration 8 mg/L was used in the study. Reverse osmosis was observed to be the most successful amongst the four methods that were used [27]. While the other processes only reduced the concentration of ibuprofen between 0.36 μg/L to 1.88 μg/L, ibuprofen was non detectable after reverse osmosis treatment [27].

2.5 KNOWN BPA AND IBUPROFEN DEGRADING BACTERIA

2.5.1 BPA degrading bacteria. Few strains of *Sphingomonas* sp. have been found to be capable of degrading BPA [43]. Strain AO 1 is a rod shaped aerobic bacterium. Strain AO 1 can use BPA as a growth substrate [43]. The cytochrome P450 system is known to be responsible for the degradation [43].

The MV1 strain isolated from a WWTP of a plastic manufacturing industry was able to utilize BPA as a sole carbon source [32]. During the degradation process several intermediates were detected. They were, 4-hydroxyacetophenone (4-HAP), 2, 3-bis (4-hydroxyphenyl)-1, 2-propanediol (BHPPD), 2, 2-bis (4-hydroxyphenyl)-1- propanol (BHPP) and 4-hydroxybenzoic acid (4-HBA) [32]. Of these metabolites, 4-HAP and 4-HBA were initially formed and were the major metabolites [32]. This was followed by the production of BHPP and BHPPD [32]. It was observed that 4-HAP and 4-HBA were able to support the growth of the cells after BPA was depleted while the other two metabolites could not [32].

BPA degrading strains were isolated from activated sludge (11 strains) and river water (8 strains) [20]. BPA removal by all the strains was 100%. In addition to the previously mentioned metabolites, 4, 4-dihydroxy-a-methylstilbene and p-hydroxyphenacyl alcohol were detected [20]. Some of the bacterial isolates were identified as belonging to *Arthrobacter, Pseudomonas* and *Enterobacteriaceae* [20].

*Streptomyces.*sp was isolated from river water in one other study and this could degrade BPA > 90% over a span of 10 days [23]. The half life of BPA degradation by *Streptomyces.*sp was found to be between 3 and 4 days [23]. The bacterial count was also found to increase over the first five days, after which there was a drop in the count till the tenth day [23].

The bacterial strains that are capable of degrading BPA are briefly summarized below in Table 5. From Table 5, it can be observed that all strains, except *Sphingomonas bisphenolica,* have utilized BPA as a sole carbon source at concentrations much higher than the environmental concentration of 0.4 μg/L [4].

TABLE 5. BPA degrading bacteria. [20, 22, 23, 32, 43]

Bacteria	Growth substrate	Conditions
Sphingomonas bisphenolica strain AO1 [43].	BPA (115 μg/L) used as sole carbon source.	BPA was added to basic mineral salts medium at 115μg/l and cultivated at 30°C in 500ml flasks in 100ml medium at 20 rpm.
Bacterial strain MV1 with ID no NRRL-B-18737 [32].	Cells were grown with BPA (342mg/L), Glucose and 4-HydroxyBenzoic Acid.	MV1 strains grown on different carbon sources were used. 25 ml of PAS basal mineral salts medium in 50 ml Erlenmeyer flasks. They were incubated at 30°C on rotary shakers at 200rpm with BPA concentration of 1.5mM.
Pseudomonas sp. and *Pseudomonas putida* strain [22].	BPA (1mg/L) used as sole carbon source.	1ml of 200mg/l BPA stock solution + 1ml working culture solution diluted to 200 ml to get a concentration of 1mg/l. Control comprised of 1ml of 200mg/l BPA stock solution + bacteria free Mueller Hilton broth diluted to 200 ml. Bottles covered with nonabsorbent cotton wool stored at 30°C.

TABLE 5. Continued.

Streptomyces sp. strain [23].	BPA (1mg/L) used as sole carbon source.	1 ml aliquot of 200mg/l BPA stock solution + 1ml working culture solution diluted to 200 ml to get a concentration of 1mg/l. Control comprised of 1ml of 200mg/l BPA stock solution + 1 ml of nutrient broth. All bottles were covered with nonabsorbent cotton wool and stored at 30°C.
Isolates belonging to *Arthrobacter, Pseudomonas* and *Enterobacteriaceae* [20].	BPA (1200mg/L) used as sole carbon source.	(i) Sludge pellets washed in 50mM phosphate buffer (pH 7.2) suspended in 50ml BPA medium and de-ionized water at 1200mg/l on shaker at 120 strokes/min at 30°C. 1 ml samples were taken, centrifuged and then the concentrations were measured. (ii) 5 ml of 10 times-concentrated river water microcosm + 45 ml of artificial river water + 1 ml of BPA solution in 100 ml Nessler tube. Incubated at 28°C in dark at 120 rpm rotary shaking. Aliquots taken at regular intervals for total organic carbon measurement.

2.5.2 Ibuprofen degrading bacteria. Another study isolated a bacterial strain from the WWTP that was capable degrading ibuprofen. The strain *Sphingomonas* sp. Ibu-2 isolated from a WWTP was able to utilize ibuprofen as a main carbon source and energy source [37]. Both the S-enantiomer and the R-enantiomer of ibuprofen were metabolized but there was a slight preference towards the R-enantiomer [37]. Isobutylcatechol was produced during the degradation of ibuprofen by *Sphingomonas* sp. Ibu-2 [37]. The propionic acid group was removed and deoxygenation at the 1, 2 positions on the aromatic ring of ibuprofen occurred [37].

A bacterial strain *Nocardia* sp. strain NRRL 4656 was also found to degrade ibuprofen [5]. Both the enantiomers were removed completely [5]. Two principal metabolites namely ibuprofenol and ibuprofenol acetate were observed during degradation [5]. The biodegradation pattern observed for both the enantiomers were slightly different [5]. In the case of the R-enantiomer, both the metabolites could be detected [5]. While in the case of the S-enantiomer, only ibuprofenol could be detected [5].

Both strains can grow on ibuprofen as the sole carbon source at concentrations ranging from 500 mg/L to 250 μg/L. However, the environmental concentrations of BPA ranged from 2 to 139 ng/L (Table 6).

TABLE 6. Ibuprofen degrading bacteria.

Bacteria	Growth substrate	Conditions
Sphingomonas sp. strain Ibu-2 [37].	Uses ibuprofen (500mg/L) as sole carbon source.	Active culture was added to 1 liter of MSM media with 500 mg of Ibuprofen and maintained for 48h-60h. Supernatant acidified to pH 3 with HCl and extracted with ethyl acetate for GCMS analysis.
Nocardia sp. [5]	Uses ibuprofen (250μg/L) as sole carbon source.	Culture grown on soyabean meal-glucose medium and ibuprofen substrate. Culture added to five1 liter DeLong flasks containing 200 ml media. 1 g ibuprofen distributed amongst the five flasks.

2.6 AMMONIA OXIDIZING BACTERIA (AOB)

2.6.1 Introduction. The AOB are chemolithoautotrophic bacteria that play an important role in the nitrogen cycle oxidizing ammonia to nitrate, the first step of nitrification [40]. The nitrification process is commonly employed in wastewater treatment [40].

They are known to corrode concrete and stonework by the production of acids, produce greenhouse gases and pollute water by nitrite and nitrate release [40]. However they are useful in the WWTPs where they are known to reduce the ammonia content [40]. This is in turn reduces the risk posed by ammonia to aquatic life, prevents eutrophication to some extent and also reduces the oxygen demand in water bodies [40].

The AOB are broadly classified into two monophyletic groups under protobacteria [21]. The AOB are known to belong to the gamma and beta subclass of protobacteria [21]. *Nitrosomonas nitrosa, Nitrosomonas marina, Nitrosomonas europaea, Nitrosomonas halophila* are some of the strains that belong to the beta class of AOB [40]. *Nitrosococcus halophilus, Nitrosococcus oceani* and *Nitrosococcus* sp. strains belong to the gamma class of AOB [40]. Extensive studies conducted on AOB have been able to identify the following diversity of AOB (Table 7) [56].

TABLE 7. Genetic diversity of AOB. [56]

Genera	**Number of species**
Nitrosomonas	10 recognized species
Nitrosococcus	3 recognized species
Nitrosospira	1 recognized species + 4 species indicated by DNA homology studies
Nitrosovibrio	1 recognized species + 1 species indicated by DNA homology studies
Nitrosolobus	1 recognized species + 1 species indicated by DNA homology studies

A study conducted on the AOB present in the activated sludge of a WWTP reported the presence of the following strains: *N.mobilis, N.europaea, N.eutropha* and *N.halophila* [21]. However *Nitrosospira* like cells were the dominant species found in the activated sludge along with *N. mobilis* [21].

Fig. 1 lists some AOB strains and their relation to the methane oxidizing bacteria.

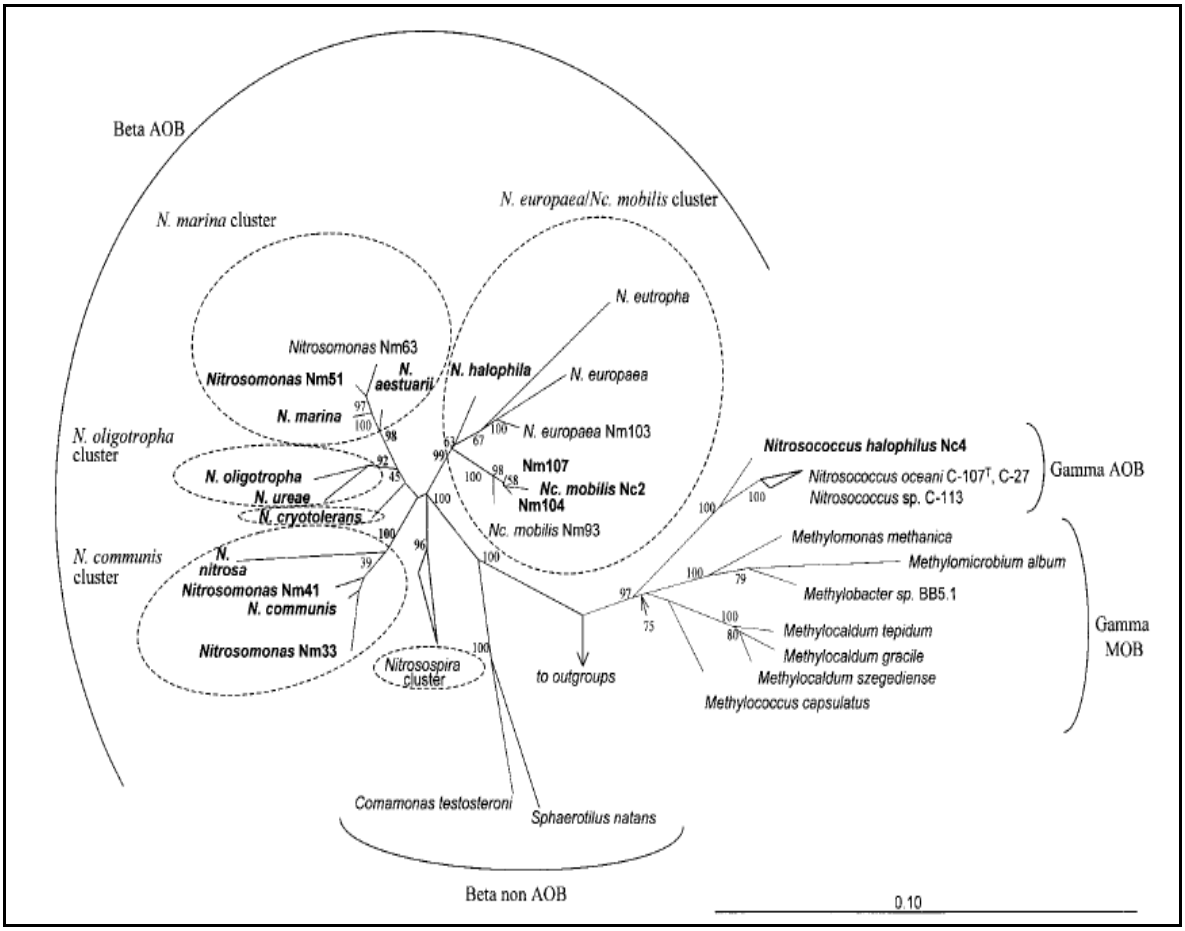


FIG. 1. AOB strains and their relationship with methane-oxidizing bacteria. [56]

2.6.2 Oxidation of ammonia. AOB utilize ammonia as an energy source [14]. AOB oxidize ammonia to nitrite in the WWTPs along with nitrifying bacteria which convert the nitrite to nitrate [14]. Since no other autotrophic bacteria are known to catalyze the oxidation of ammonia to nitrite, AOB play a very important role in the nitrification process in WWTPs [14].

The conversion of ammonia to nitrite is carried out by principally two enzymes namely hydroxylamine oxidoreductase (HAO) and ammonia monooxygenase (AMO) [14]. HAO and AMO enzymes work in tandem to bring about the oxidation of ammonia [14].

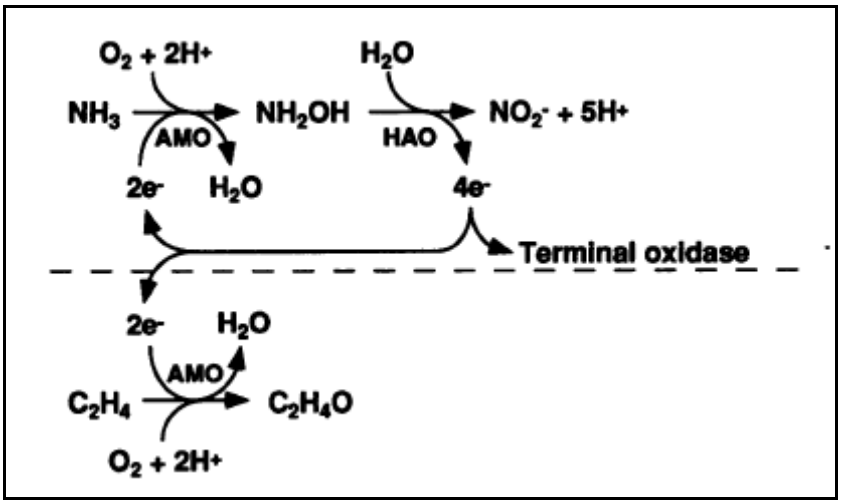


FIG. 2. Reactions catalyzed by AMO and HAO. "AMO- ammonia monooxygenase, HAO- hydroxylamine oxidoreductase, P460- cytochrome P460, Q- ubiquinone-8, CycB- teraheme membrane c-cytochrome, c552- cytochrome c552, ccp- diheme c553 peroxidase, NiR- nitrite reductase, CuCuaa3- cytochrome oxidase, NOR- nitric oxide reductase, N_2OR – nitrous oxide reductase. Solid arrows show known pathways. Dashed arrows show hypothesized pathways" [24].

The oxidation of ammonia to hydroxylamine is initiated by two electrons that are brought to AMO [14]. The hydroxylamine is then oxidized to nitrite with the release of four electrons [14]. The four electrons are transferred by the HAO to cytochrome 554 (c554) which is the sole source of energy transfer and biosynthesis for AOB [14]. Out of these four electrons, two are used for the regeneration of hydroxylamine and the other two electrons are used in the reduction of pyridine nucleotide [14]. The leftover electrons are passed on to cytochrome aa3 reductase from c554 or used in the NO and N_2. The reduction is carried out by NO and N_2O reductases [14].

2.6.3 Ammonia monooxygenase. AMO plays an important role in the oxidation of ammonia (Fig. 2). AMO is a membrane bound enzyme and oxidizes ammonia to

hyrdroxylamine which is further oxidized to nitrite by HAO [24]. Similar to methane monooxygenase, AMO is also known for the wide substrate range it catalyzes [24]. AMO is known to degrade aromatic hydrocarbons, alkanes, alkenes and chlorinated hydrocarbons [24].

Ammonia serves as the substrate for the enzyme as compared to ammonium ions [14]. The substrates or competitive inhibitors for the AMO enzyme are mostly non-polar in nature (Fig. 3) [14]. The different kinds of compounds in the substrate range suggest the activity of the enzyme is triggered by oxygen rather than the substrate [14]. The oxygen from the AMO enzyme is highly reactive and is known to react with the N-H bonds present in ammonia, C-H bonds present in methane and other hydrocarbons [14]. The reactions concerning AMO are of two broad types. The AMO enzyme either initiates a dehydrogenase or oxidase reaction or a reductive dehalogenation reaction [14].

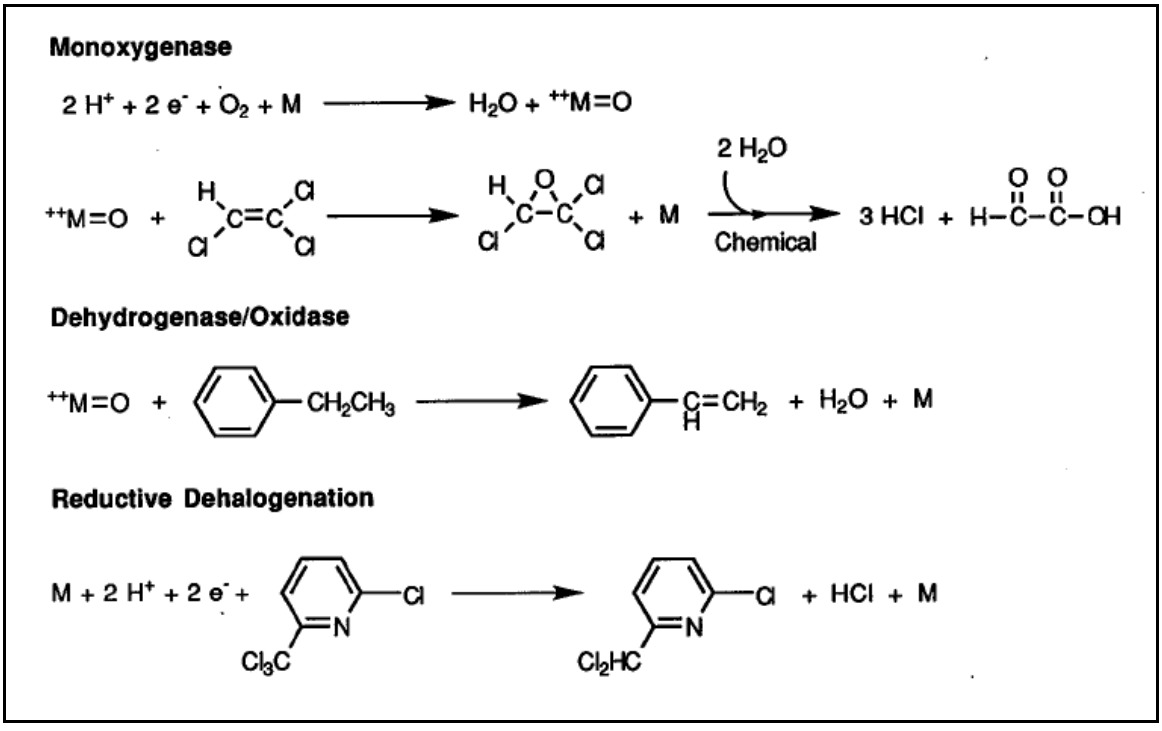


FIG. 3. Types of reactions characterized by AMO. [14]

At very low oxygen levels, significant amounts of nitrous oxide, nitric oxide and nitrogen gas are produced by AOB [14]. In such cases mostly nitrite is the electron acceptor and compounds like pyruvate and hydrogen act as reductants [14]. The growth of the organisms under such conditions was observed to be slower than aerobic conditions [14].

2.6.4 ***Nitrosomonas europaea.*** *N.europaea* belongs to the β-subdivision of the protobacteria which is derived from photosynthetic bacteria [28]. Most of the other *Nitrosomonas* and *Nitrospira* strains belong to the β- subdivision [28]. *N.europaea* with other AOB takes part in the nitrification process which is an important step in the biogeochemical nitrogen cycle [4]. However, some by-products of this cycle namely gases like nitric oxide and nitrous oxide are essentially green house gases and play a role in global warming [4]. But the activity of the microorganisms also provides nitrogen to plants and is used in waste water treatment and remediation of contaminated sites [4].

N.europaea obtains the energy and reductant required for its growth by the oxidation of ammonia [4]. The main carbon source for the cell is carbon dioxide [4]. So far, it has been observed that *N.europaea* is not capable of utilizing any other organic or inorganic compound as a source of its energy [4]. The study of the bacterium's genome did not show any evidence for the reduction of Fe, CO, H_2 or other sources of energy [4]. A study has also reported a loss in the activity of *N.europaea* due to the availability of limited ammonia or ammonium ion [51].

Studies have also reported the wide substrate range of *N.europaea* [6, 25]. Due to the AMO enzyme, they are known to catalyze the oxidation of many different aromatic and aliphatic organics via co-metabolic degradation (non beneficial mechanism) [6, 25].

In this study, it is hypothesized that BPA and ibuprofen can be cometabolically degraded during the oxidation of ammonia to nitrite (Fig. 4).

Some of the hydrocarbons that lie in the substrate range of *N.europaea* are outlined in Table 8:

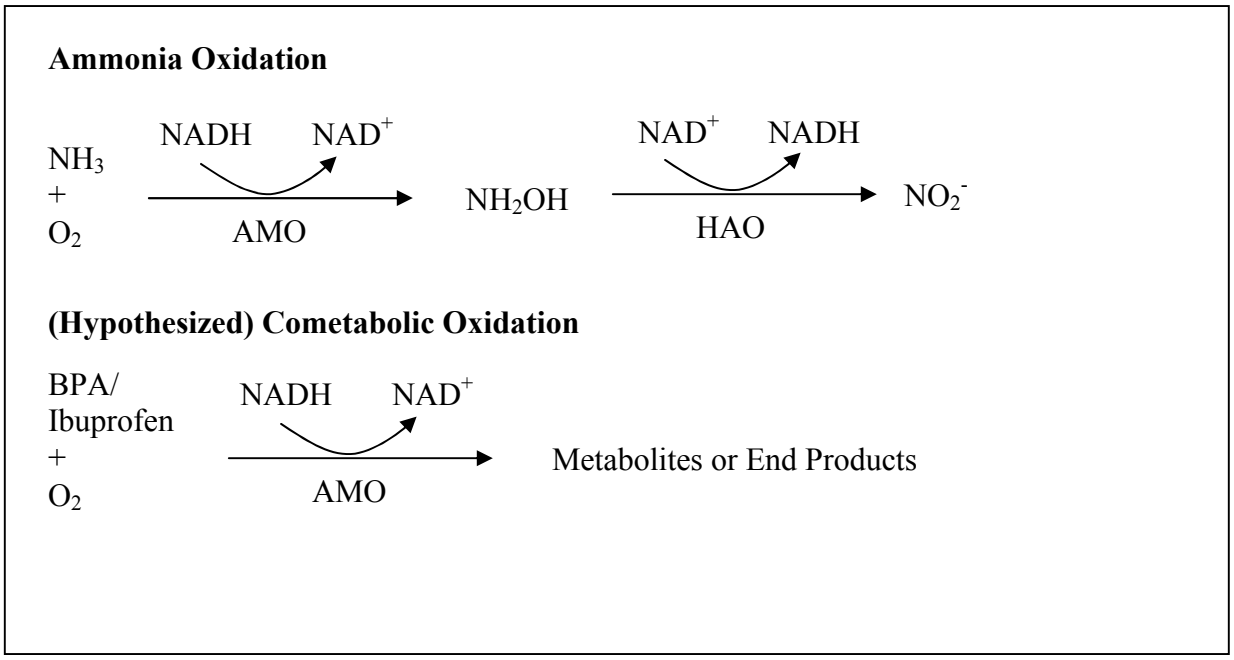


FIG. 4. Hypothesized cometabolic reactions by *N.europaea.*

TABLE 8. Oxidation of n-alkanes and n-alkenes by *N.europaea.* [25]

Substrate (n-alkanes)	**Product**
Methane	Methanol
Ethane	Ethanol
Propane	Propan-1-ol, propan-2-ol, both.
Butane	Butan-1-ol, butan-2-ol, both
Pentane	Pentan-1-ol, pentan-2-ol, both
Hexane	Hexan-1-ol, hexan-2-ol, both
Heptane	Heptan-1-ol, heptan-2-ol, both
Octane	Octan-1-ol, octan-2-ol
Substrate (n-alkenes)	**Product**

TABLE 8. Continued.

Ethylene	Ethylene oxide
Propylene	Propylene oxide, 2-propene-1-ol, both
1-butene	1,2-epoxybutane, 3-butene-1-ol, 3-butene-2-ol, all the three
1-pentene	1,2-epoxypentane, 4-pentane-1-ol, 4-pentane-2-ol, all the three
2-butene (*cis*)	*Cis* 2,3-epoxybutane, *cis* 2-butene-1-ol, both
2-butene (*trans*)	*Trans* 2,3-epoxybutane, *trans* 2-butene-1-ol, both

Several other aromatic compounds like benzene, phenol, halogenated aromatic compounds like chlorobenzene, bromobenzene, aromatic alcohols like benzyl alcohols, cresols, aromatic compounds with nitrogen and oxygen like aniline, acetophenone and so on are also degraded by *N.europaea* [25]. Recently is has been reported that *N.europaea* can also degrade estrogens namely estrone, 17 β- estradiol, estriol and 17 α-ethynylestradiol [46]. Intermediates were also detected at the end of this process and it was observed that *N.europaea* was unable to degrade these intermediates [46].

2.7 COMETABOLISM

2.7.1 Introduction. The biological processes that take place during the degradation of organic compounds are primarily of two types. The first kind of process occurs when the organic compounds are used as the primary substrate or they are the main carbon and energy source [35]. The second type of reaction is named cometabolism [35].

Cometabolism is an important process that takes part in the degradation of several toxic compounds in the environment. According to the EPA website cometabolism is defined “as a process in which there is a simultaneous metabolism of two compounds such that the degradation of the second compound depends on the presence or

degradation of the first or the primary compound" [6]. Cometabolism occurs by the action of enzymes that are produced by the microorganisms primarily for some other purpose [35]. Cometabolism can hence be termed as a "fortuitous reaction" [35].

Cometabolism may or may not be beneficial to the microorganisms. In some cases it can be harmful to the microorganism [35]. Cometabolism can occur if there are sufficient numbers of microorganisms to produce the required enzymes [35]. Another requirement is the presence of the primary substrate which is the main carbon and energy source [35]. It is necessary that the substrate should be present in sufficient quantity to be able to overcome the toxic effects occurring during cometabolism [35]. In several cases this process requires additional energy that is provided by a complex enzymatic reaction in the form of NADH or NADPH [35].

Many monooxygenases and dioxygenases can oxidize many different compounds through cometabolic reactions [35]. Many oxygenase-expressing microorganisms, like methane oxidizers, toluene oxidizers, ammonia oxidizers and phenol or cresol oxidizers are known to catalyze cometabolic reactions [35]. However the microbial behavior of the above organisms differs with different organisms [35]. The methane oxidizers that express methane monooxygense enzyme are currently one of the most studied micro organisms [35].

2.7.2 Competitive inhibition. Competitive inhibition occurs when two compounds are oxidized by the same enzyme. Hence, primary and cometabolic substrates become competitive substrates [35]. For example, methane monooxygenase (MMO) can oxidize both methane and tri-chloro ethylene (TCE). Hence methane and TCE competitively inhibit each other [35].

Competitive inhibition can be overcome by supplying the primary substrate at regular intervals, just sufficient to induce the enzyme activity [35]. Another way is to grow the microorganism on a primary substrate to induce the enzyme and then adding secondary substrate to prevent competitive inhibition [35].

2.7.3 Transformation product toxicity (TPT). One of the major factors that hinder cometabolism is the transformation product toxicity (TPT) [35]. During the course of the

reaction certain intermediate products are formed which are toxic to the microorganisms and hence reduce the activity of the cell [35]. It can have the following repercussions:

(1) Inactivation of the enzyme

(2) Interacts with other cellular components and damages it

(3) Death of the cell

Transformation capacity (T_c), a measure of TPT is defined as the amount of compound that can be transformed before cell inactivation [35].

CHAPTER III
MATERIALS AND METHODS

3.1 CHEMICALS

BPA (99% purity) was obtained from TCI America, Inc., Portland, OR. Ibuprofen (USP grade) was obtained from MP Biomedicals, Aurora, OH. Stock solutions (1000, 100, 10, 1 and 0.1 mg/L) for both Ibuprofen and BPA were prepared in acetone. The standards were prepared in 40 ml glass vials. Ethyl ether was purchased from Fisher Scientific, Inc., Fair Lawn, NJ. Allythiourea, an inhibitor for the AMO enzyme activity [45], was bought from Sigma-Aldrich, Inc., St. Louis, MO. Dimethylformamide (DMF) and *N,O-bis*(trimethylsilyl)-trifluoroacetamide (BSTFA) were purchased from Pierce Biotechnology, Inc., Rockford, Il. Tetrazotized o-anisidine was purchased from Fluka chemicals, Sigma-Aldrich, Inc., St. Louis, MO.

3.2 CULTURE AND GROWTH CONDITIONS

3.2.1 AOB (pure culture). *Nitrosomonas europaea* culture was provided by Dr. Michael R. Hymann, Department of Microbiology, North Carolina State University. The culture was grown in sterilized 2L flasks containing 1000ml of a mineral salts medium [$NH_4(SO_4)_2$, 3.3 g, 0.33g, 0.66g; KH_2PO_4, 0.4L; NaH_2PO_4, 0.48g; 1M $MgSO_4$, 0.75ml; 1 M $CaCl_2$, 0.2ml; 30mM $FeSO_4$/ 50mM EDTA stock solution, 0.33ml; 50mM $CuSO_4$, 0.01 ml; KH_2PO_4, 5.44g; Na_2CO_3, 0.04% in 1000ml] [17]. The flasks were incubated at 180 rpm at 30°C in the dark. Na_2CO_3 (0.003M) was the sole carbon source. The culture was allowed to grow for 3 days before harvesting for experimental use.

3.2.2 AOB (mixed culture). The activated sludge samples were collected from the Carter's Creek WWTP located in College Station and the 36th street WWTP belonging to the City of Houston.

3.2.2.1 Activated sludge (combined reactor). The activated sludge from the Carter's Creek WWTP located in College Station was collected from a single reactor that was employed for both carbon and nitrogen removal. The single stage reactor was designed for an average flow of 2.5 million gallons per day (MGD) while the peak design flow

was 7.8 MGD. However the average flow through the aeration basin was recorded to be 1.5 MGD. The WWTP consisted of four different aeration basins with a length of 88 feet, width of 33 feet and a depth of 12 feet. Each aeration basin consisted of two compartments each with each compartment containing two grids. The influent and the return activated sludge entered through the grid nearest to the influent through the various compartments of all the aeration basins through holes in the walls between chambers. A total of 2,724 grid (ceramic fine bubble) diffusers were distributed over the grids with the highest numbers in the grid nearest to the influent. The aeration basin was fed through a single influent inlet. The average influent biological oxygen demand (BOD) and MLVSS were 202 mg/L and 2057 mg/L respectively. The average influent and effluent ammonia concentration was 22 mg/L and 0.35 mg/L. The sludge retention time varied between 7 to 12 days.

3.2.2.2 Activated sludge (nitrifying reactor). The Houston WWTP employed a two step treatment process where the first step was the carbonaceous treatment and the second step was nitrogenous treatment. The WWTP had eight parallel treatment trains with each trains consisting of a first step reactor (carbonaceous) and a second step reactor (nitrogenous). The carbonaceous reactor (116 feet x 116 feet) consists of four compartments (58 feet x 58 feet) with an average side water depth of 14.8 feet arranged in series. Each cell had one mixer and a power blower. The second step reactor consisted of a reactor (133 feet x 133 feet) with the three compartments; first compartment (66 x 133 feet), second and third compartment (66 x 66 feet) with an average side water depth of 14.8 feet. The first compartment had two mixers and a purge blower while the second and third compartment had one of each.

Each reactor was fed independently from the same source with flow splitter box. Each reactor was designed to treat an average flow 25 MGD and a peak flow of 50 MGD. The influent average temperature, BOD and MLVSS were recorded as 25°C, 165 mg/L and 70 %. The average influent and effluent ammonia was 17 mg/L and 2.8 mg/L. The carbonaceous reactor employed 1 day old sludge while the nitrogenous reactor used 30 days old sludge.

3.3 DEGRADATION TESTS WITH PURE CULTURE

The degradation tests were conducted in 1000 ml flasks with different volumes for different experiments. Appropriate amounts of the compound (BPA and ibuprofen) were added from the stock solutions that were previously prepared. BPA was added along with the acetone while acetone was evaporated in the case of ibuprofen. Experiments conducted earlier with acetone evaporation resulted in very low concentrations of BPA. BPA and ibuprofen were then allowed to dissolve over 3 days in a dark room since BPA is photosensitive. Due to the low detection limit of the GCMS, higher experimental concentrations of BPA and ibuprofen were used as compared to the environmental and toxicity concentrations [3, 10, 30, 34, 39, 42, 44].

The pre-cultivated culture was centrifuged at a speed of 167 rps to spin down most of the cell suspended in the media. The cell was then washed using a phosphate buffer solution (0.05M NaH_2PO_4 and 0.002M of $MgSO_4$) for one of the experiments. The cell was further re-suspended in the media and added to the 1000ml bottles containing BPA or ibuprofen. The optical density in the 1000ml bottles was maintained at 0.076 or 0.4 using a spectrophotometer (Agilent 8453, USA) at 600nm. The corresponding MLVSS for the two optical densities was 229 mg/L and 1385 mg/L. To achieve faster degradation, in stead of using the lower cell mass, a higher cell mass was used in the later experiments

The experiments were conducted in different conditions in the 1000 ml flasks. The activity of AMO enzyme was inhibited by adding an AMO inhibitor, allylthiourea (10 mg/L), into two of the flasks. Sodium formate (20mM) was added to two additional flasks to provide reducing energy for the biodegradation process. Sterilized cells were used for the sterile control for the purpose of abiotic activity. The 1000ml flasks were then kept on a shaker for thorough mixing at 180 rpm at 30°C in the dark. Samples were drawn at regular intervals for analyses.

The initial experiment was conducted to examine if BPA could be degraded by *N.europaea.* A similar experiment was conducted with resting cells. Both the experiments and the results are described in detail in the appendix.

3.3.1 Experiments on BPA.

3.3.1.1. BPA (0.75 mg/L). A BPA concentration of 0.75 mg/L and a final volume of 150 ml was used for the experiment. The *N.europaea* culture for the experiment was grown on two different ammonia concentrations of 5mM and 10mM. This was done to compare the differences in the degradation of BPA by cells grown on different ammonia concentrations. The cells grown on 5mM ammonia were suspended in media containing 5mM ammonia and the cells grown on 10mM ammonia were suspended in media containing 10mM ammonia. The cells were maintained at a higher optical density of 0.4 (1,385 mg/L) for the current experiment. The experiment excluded the addition of sodium formate and inhibitor to the flasks.

3.3.1.2. BPA (1 mg/L). This experiment was conducted with BPA (1 mg/L) and the final volume was 150 ml. The cells for the experiment were grown on an ammonia concentration of 10mM and with an optical density of 0.4 (1385 mg/L).

3.3.2 Experiments on ibuprofen. The experiments on ibuprofen were conducted for two concentrations of 0.8 mg/L and 0.4 mg/L (Appendix I). For both the experiments an optical density of 0.4 (MLVSS = 1385 mg/L) and a final volume of 150 ml was used. The cells were grown on an ammonia concentration of 10mM.

3.4 DEGRADATION TESTS WITH ACTIVATED SLUDGE

The experiments with the activated sludge were conducted on BPA (1mg/L) and ibuprofen (0.5 mg/L). The activated sludge in the first experiment was obtained from a combined reactor with both oxidation and nitrification taking place in the same reactor. For the second experiment the activated sludge was obtained from the nitrification tank.

The experiment was conducted on similar lines as the pure culture. All the experiments were conducted with the compounds BPA and ibuprofen, with and without the presence of inhibitor allylthiourea and by using sterilized activated sludge for the kill control. The addition of AMO inhibitor would allow for examining whether AMO enzymes play a role on BPA and/or ibuprofen biodegradation. The MLVSS of the sludge was first measured and adjusted to 1000 mg/L in a final volume of 150 ml and 300 ml.

The sludge was washed with a phosphate buffer solution (0.05M NaH_2PO_4 and 0.002M of $MgSO_4$) after centrifuging it.

3.5 BPA AND IBUPROFEN ANALYSIS

5 ml samples were collected from the 1000ml flasks over 30 hours. Samples were collected every 24 hours for the mixed culture experiment. For the pure culture experiments samples at shorter intervals were collected. The first sample was always collected at 0 hours. 100 µl of concentrated H_2SO_4 (17.8 M) was added to each of the samples to stop microbial activity. The samples were then stored at 4°C.

The samples were diluted by the addition of 5 ml of DI water before ether extraction. By adding 10 ml ethyl ether to the samples, BPA and ibuprofen were extracted from ether. For complete extraction, the samples were placed on the shaker for 24 hours at the high speed setting. Extracted 500 µl sample along with the standards prepared in acetone were evaporated overnight. 450 µl of DMF and 50 µl of BSTFA were added to derivatize the samples for two hours.

The derivatized samples were injected into an Agilent Technologies 6890N Network Gas Chromatography System (Santa Clara, CA) consisting of a HP-5MS column (30.0m x 250µm (i.d) and 0.25µm thickness), an Agilent 5873 Network mass selective detector and an Agilent MSD ChemStation. Helium is used as the carrier gas at 40 cm/s. The injector temperature is 250°C. The oven temperature is initially set at 80°C and then ramped at 30°C/min to 280°C and held for 3 min. The selective ion monitoring mode was used with the selected ions of 357 *m/z* and 372 *m/z* for BPA and 263 *m/z* and 278 *m/z* for ibuprofen. The detection limit for BPA and ibuprofen was 0.01 mg/L.

3.6 NITRITE AND NITRATE ANALYSIS

Samples of up to 7-10ml were collected for ammonia, nitrate and nitrite analyses. The samples were filtered and stored at -20°C before use. The standard solutions ranging from 1 mg/L to 300 mg/L were prepared using sodium nitrite and sodium nitrate in DI

water as the detection limit of the instrument was 1 mg/L. Samples of 10µl were injected for analysis to a DIONEX, DX-80 Ion Chromatograph (IC) (Sunnyvale, CA) equipped with an IonPac AS14A- 5µm analytical column (3 x 150 mm) for anion separation. An eluent solution of 0.16M Na_2CO_3 and 0.02M $NaHCO_3$ was used at a flow rate of 0.5 ml/min.

3.7 AMMONIA ANALYSIS

The ammonia analysis was conducted on an Accumet SN5244072P ammonia selective probe (Houston, TX). The standard solutions were prepared with ammonium sulphate in DI water from 1 mg/L to 500 mg/L. The detection limit of the instrument was 1 mg/L. 5ml of the samples were diluted to 50ml. 500 µl of 10N NaOH was added to the sample in order to convert NH_3 to NH_4^+ ion. The concentration of ammonia was determined by comparing to the standard curves.

3.8 NAPHTHALENE OXIDATION TEST

Naphthalene oxidation tests were conducted as described by Chu and Alvarez-Cohen [16] to measure non-specific monooxygenase activity. The teat was based on the fact a non-specific monooxygenase like AMO can oxidize naphthalene to 1- or 2-naphthol which can interact with tetrazotized o-anisidine to produce purple color [16].

Naphthalene was prepared by adding naphthalene crystals in DI water and placing it on the shaker for over 24 hours. The tests were conducted in vials by adding 1 ml samples, 1 ml of naphthalene solution and 1 ml of sodium formate (20mM). The vial was then incubated in the dark at 30°C at 180 rpm for one hour. After adding 100 µl of tetrazotized o-anisidine 0.2% (w/v) solution, the optical density was measured at a wavelength of 530 nm by using an Agilent 8453 photospectrometer (Englewood, CO). The measurements were made within two minutes after the addition of the tetrazotized o-anisidine solution as the absorbance is known to increase beyond two minutes. The controls were prepared without cells. The blanks were prepared by adding DI water and cells.

CHAPTER IV

RESULTS

This study was conducted to assess the degradation capability of BPA and ibuprofen by *Nitrosomonas europaea*. Experiments were designed with an AMO inhibitor (allylthiourea), addition of reducing energy (sodium formate) and/ or different ammonia concentrations (5mM and 10mM).

4.1 BIODEGRADATION OF BPA AND IBUPROFEN BY *N.europaea*

4.1.1 Effects of ammonia concentration on BPA degradation. Experiments were conducted on BPA (0.75 mg/L) using cells grown on ammonia of concentration 5mM and 10 mM. BPA was removed completely by 3.5 hours for 10 mM ammonia (Fig. 4-1a) and in 48 hours for 5 mM ammonia (Fig. 4-1a). Experiments were also conducted with resting cells which are explained in Appendix I.

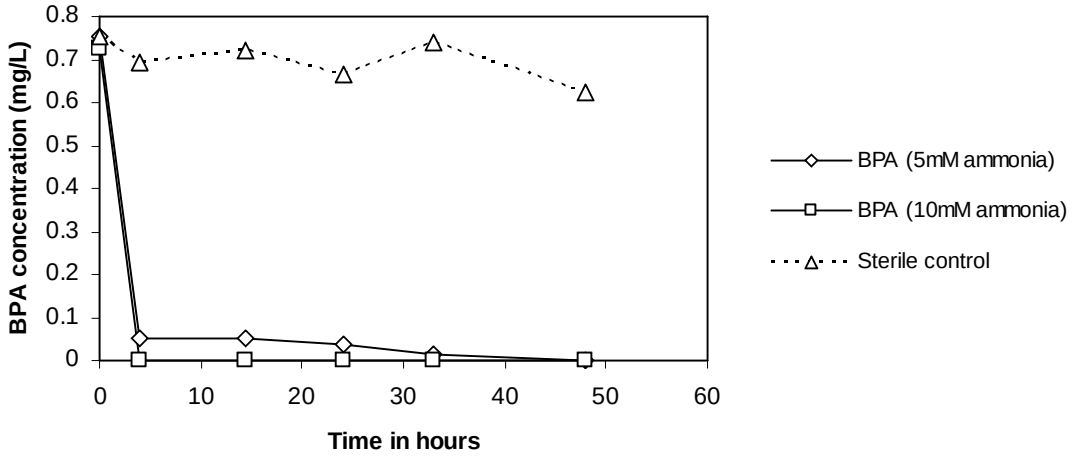


FIG. 4-1a. Degradation of BPA (0.75 mg/L) with ammonia concentrations of 5mM, 10mM.

The cell activity with respect to nitrite production was higher (0.14 absorbance) for 10 mM ammonia while it was lower (0.078 absorbance) for 5 mM ammonia (Fig. 4-1b). The nitrite production and ammonia oxidation was completely achieved within 3.5 hours (Fig. 4-1c and d), regardless of the ammonia concentrations.

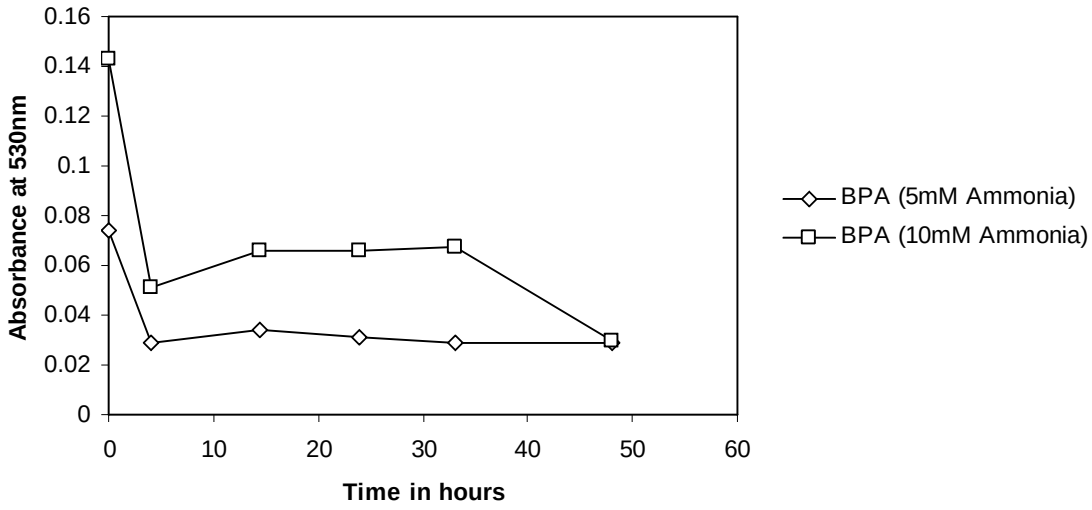


FIG. 4-1b. Naphthalene oxidation assay for cells grown on 5mM, 10mM ammonia and BPA 0.75 mg/L.

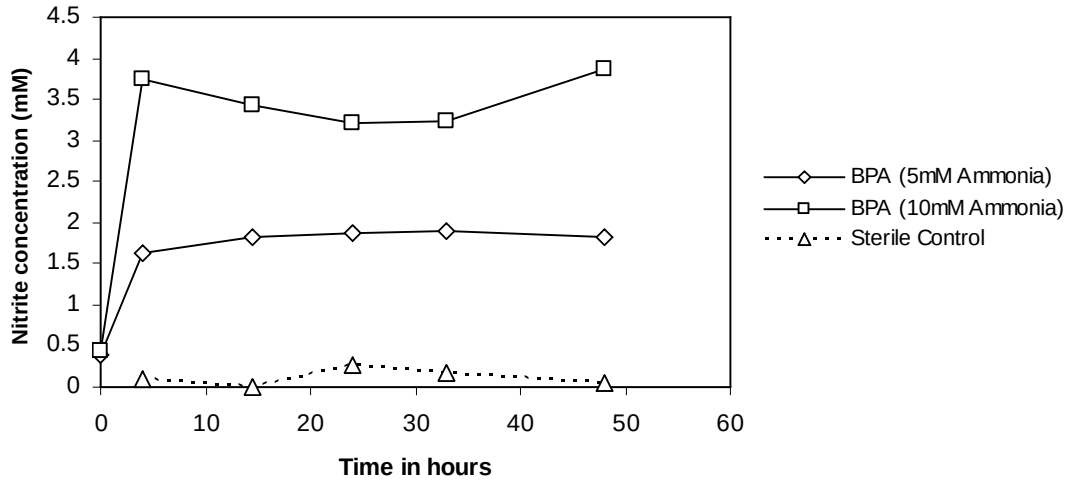


FIG. 4-1c. Production of nitrite by cells grown on 5mM, 10mM ammonia with BPA (0.75 mg/L).

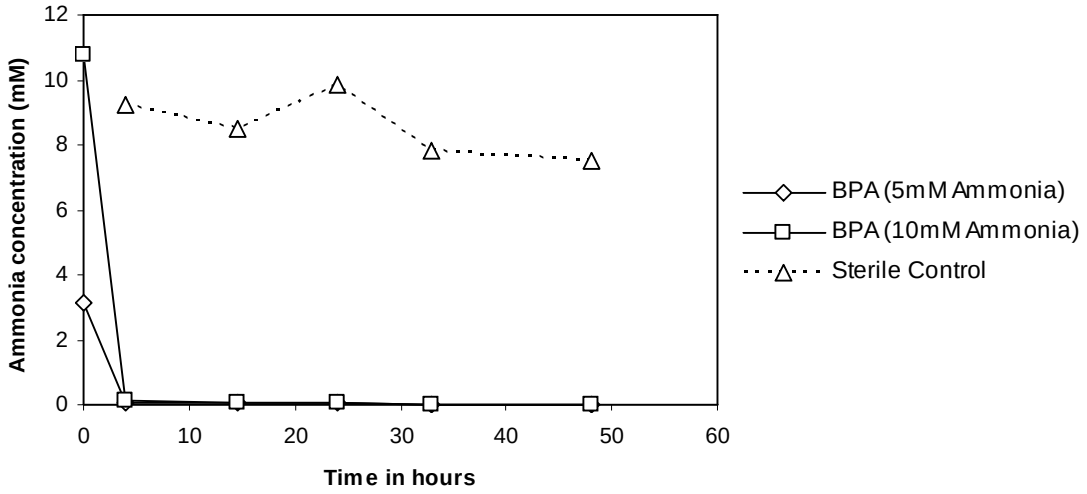


FIG. 4-1d. Oxidation of ammonia by cells grown on 5mM, 10mM ammonia with BPA (0.75 mg/L).

4.1.2 Cometabolic degradation of BPA by AMO. The experiment was conducted with two BPA concentrations of 1.6 mg/L with lower cell mass (Appendix I) and 1 mg/L with higher cell mass. The experiment was conducted with *N.europaea* grown on 10mM ammonia with the optical density (OD) adjusted to 0.4 (1385 mg/L).

BPA was degraded from 1 mg/L to 0.2 mg/L confirming the results from the previous experiment as explained in section 4.1.1. The AMO inhibitor successfully inhibited the reaction as there was no reduction in BPA concentration (Fig. 4-2a). The decrease in the cell activity can be observed from the naphthalene oxidation assay conducted (Fig. 4-2b).

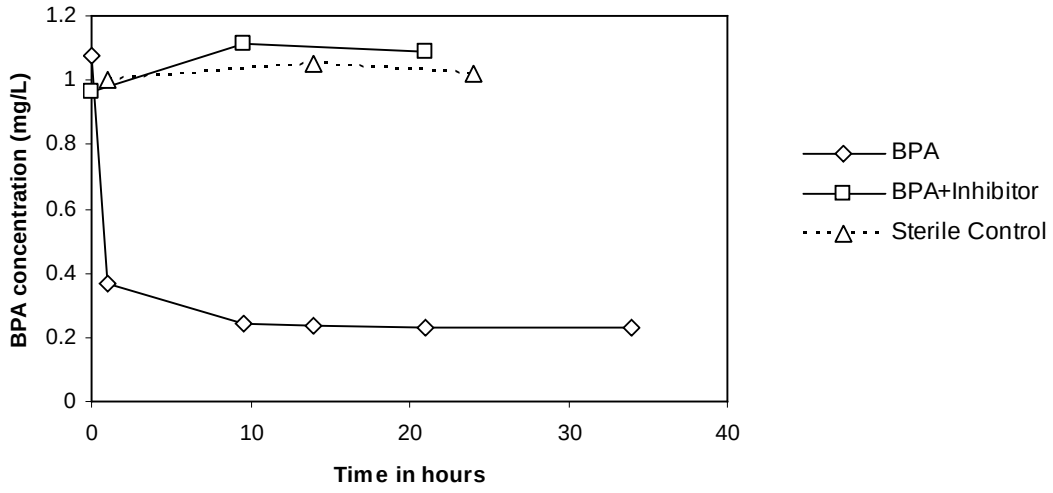


FIG. 4-2a. Degradation of BPA (1 mg/L) with and without AMO inhibitor allylthiourea.

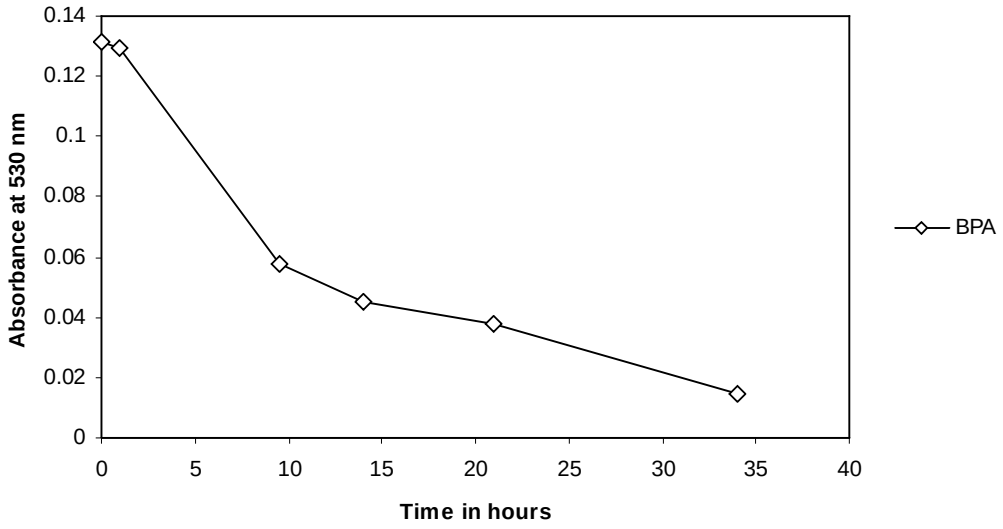


FIG. 4-2b. Naphthalene oxidation assay for BPA (1mg/L) and optical density of cell 0.4.

The nitrite production and ammonia oxidation can be seen in Fig 4-2c and Fig 4-2d.

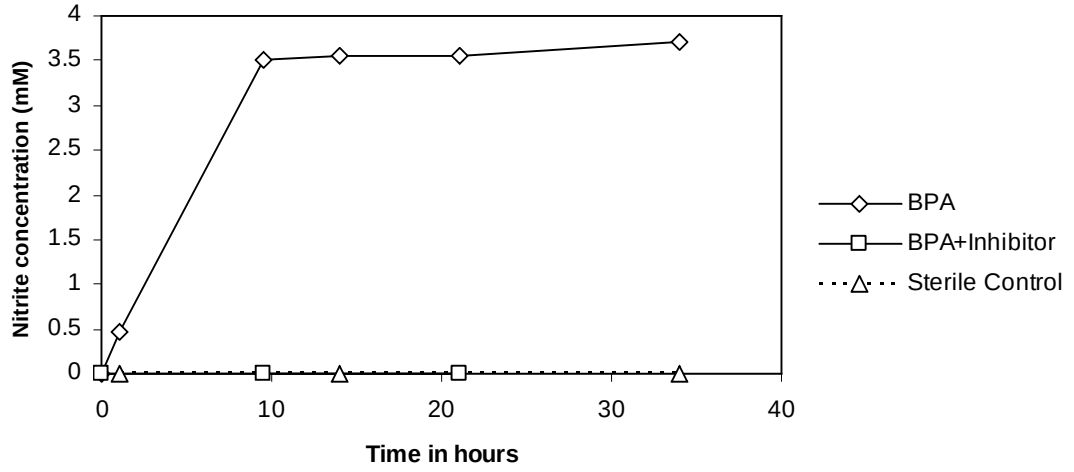


FIG. 4-2c. Nitrite production for BPA (1 mg/L) with initial ammonia concentration of 10mM.

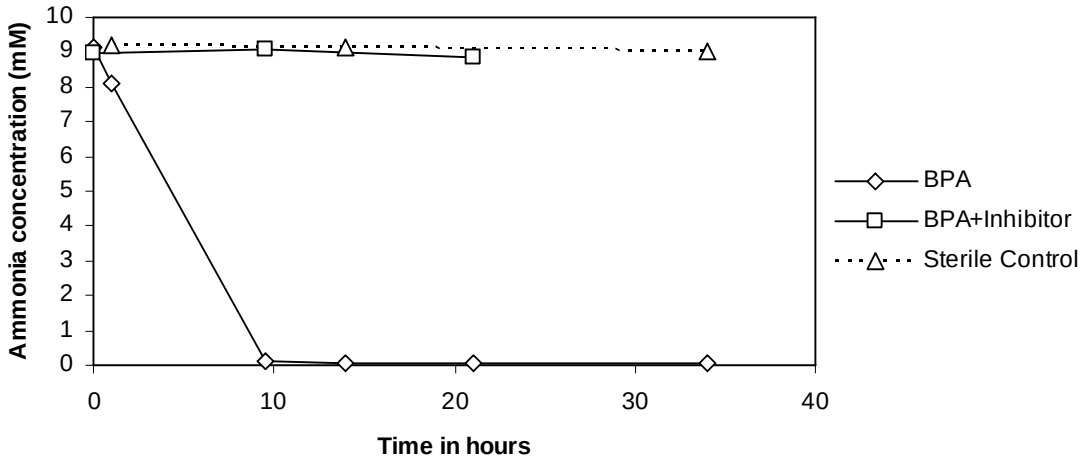


FIG. 4-2d. Ammonia oxidation with BPA (1 mg/L) and initial ammonia concentration of 10 mM.

4.1.3 Cometabolic degradation of ibuprofen by AMO. The experiments on ibuprofen were conducted on ibuprofen of concentrations 0.8 mg/L and 0.42 mg/L (Appendix I). The cells were grown on 10 mM ammonia and adjusted to an OD of 0.4. There was no degradation of ibuprofen observed as seen in Fig. 4-3a.

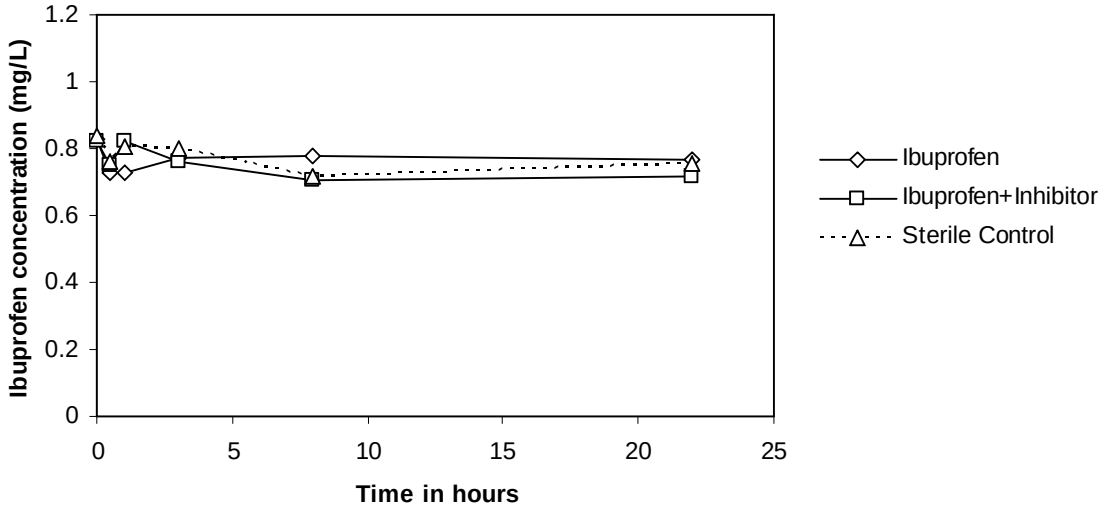


FIG. 4-3a. Degradation of ibuprofen of concentration 0.8 mg/L.

From Fig. 4-3b, it was found that the absorbance (cell activity) was very low (0.01) for ibuprofen of 0.8 mg/L.

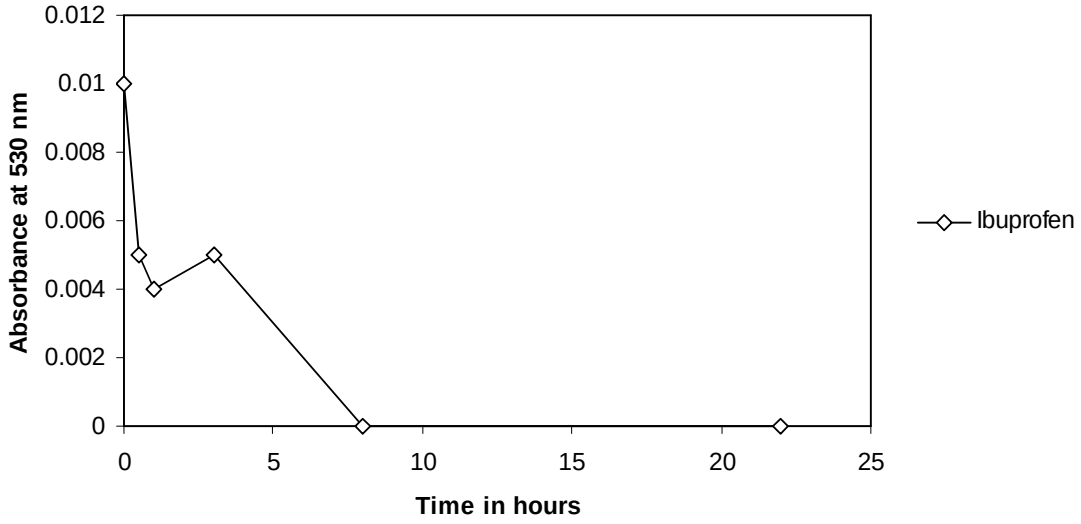


FIG. 4-3b. Naphthalene oxidation assay for ibuprofen of 0.8 mg/L with an initial ammonia concentration 10 mM.

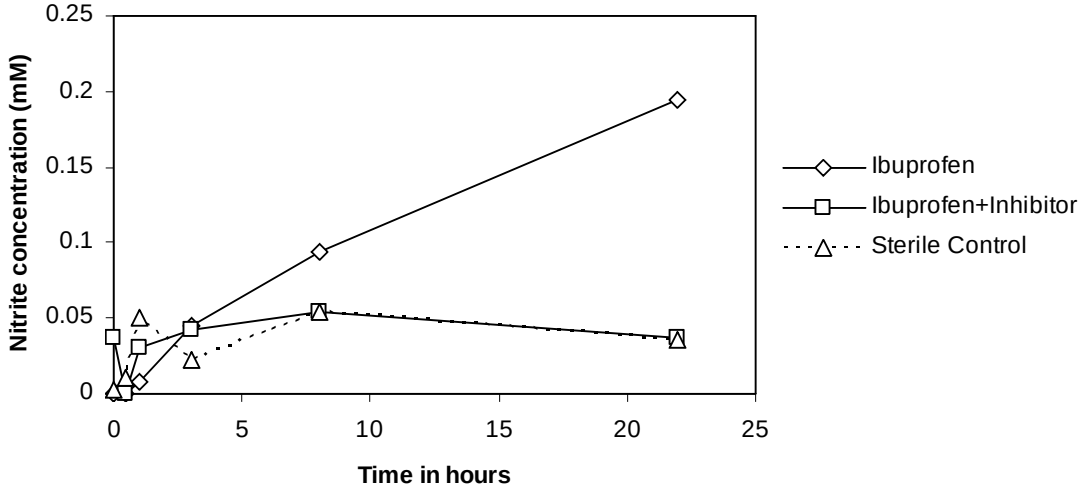


FIG. 4-3c. Nitrite production with initial ibuprofen concentration of 0.8 mg/L, the culture grown on 10mM ammonia.

The nitrite production and ammonia oxidation was < 10 mg/L (Fig. 4-3c and d) corresponding to the low AMO activity.

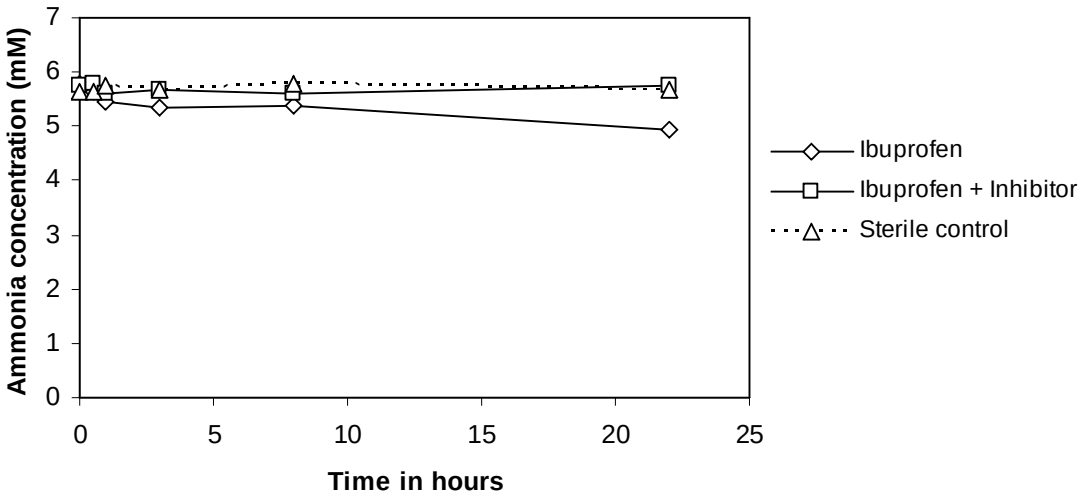


FIG. 4-3d. Ammonia oxidation by *N.europaea* grown on 10mM ammonia with initial BPA concentration of 0.8 mg/L

Table 9 contains the rates of degradation for the experiments conducted on the BPA degradation. The rates were calculated by dividing the difference in concentration between the 0 hour sampling point and the next sampling point (4 hours and 1 hour) over time. In order to normalize the degradation rates the rates so obtained were divided by the cell mass used for the experiments. The highest rate of degradation was observed for the cells grown on 10 mM ammonia and BPA of concentration 1 mg/L.

TABLE 9. Degradation rates of BPA by pure culture.

Experiment	**Initial specific degradation rate (mass BPA/ mass MLVSS x h)**
BPA of concentration 0.75 mg/L with MLVSS 138.5 mg/L and ammonia concentrations of: 1. 5 mM 2. 10 mM	 1. 1.27 E -04 2. 1.35 E -04
BPA of concentration 1 mg/L with MLVSS 1385 mg/L and initial ammonia concentration of 10 mM	5.13 E -04

4.2 BIODEGRADATION OF BPA AND IBUPROFEN BY ACTIVATED SLUDGE (MIXED CULTURE)

4.2.1 Biodegradation by activated sludge from one stage aeration tank. BPA (1 mg/L) and ibuprofen (0.5 mg/L) were subjected to degradation by activated sludge from a reactor that was designed for both oxidation and nitrogen removal. The BPA was degraded from 1 mg/L to 0.2 mg/L in the presence and absence of the inhibitor allylthiourea (Fig. 4-4a). However ibuprofen was removed completely at the end of the 2^{nd} day (Fig. 4-4b).

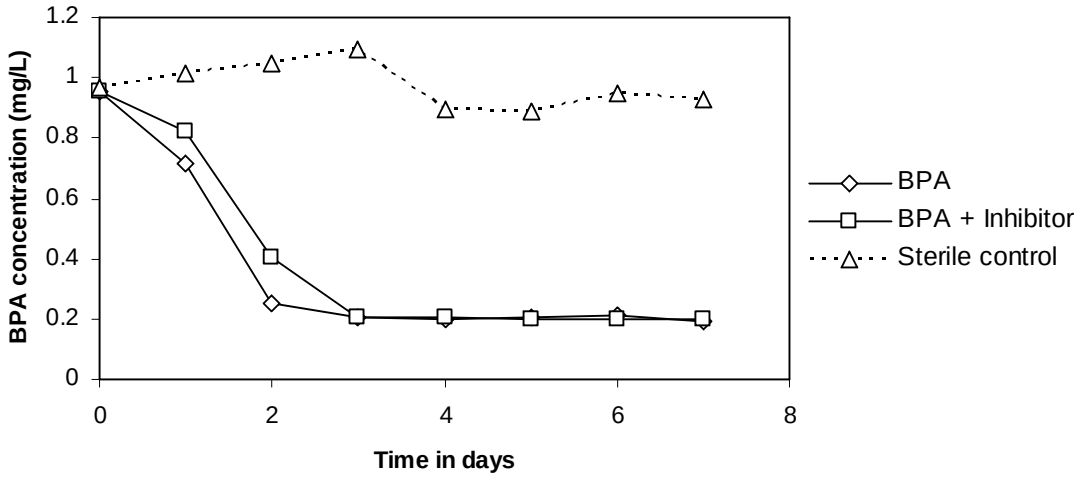


FIG. 4-4a. Degradation of BPA (1 mg/L) by activated sludge from a single stage aeration tank.

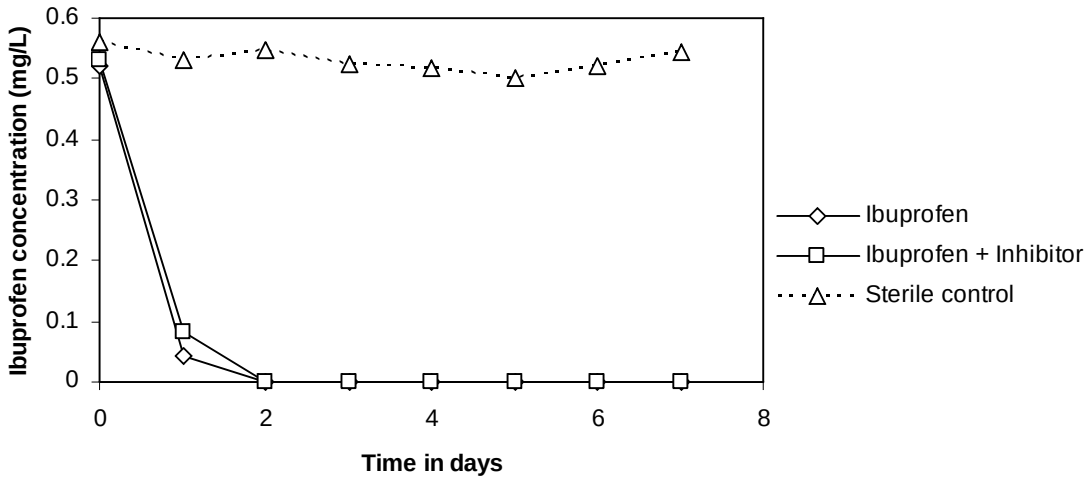


FIG. 4-4b. Degradation of ibuprofen (0.5 mg/L) by activated sludge from a single stage aeration tank.

There was no production of nitrite but nitrate production commenced on the 6th day for BPA and on the 5th day for ibuprofen (Fig. 4-4c and d). Ammonia oxidation was observed after the 2nd day for BPA (Fig. 4-4e) while the ammonia oxidation was completed by the 2nd day for ibuprofen (Fig. 4- 4f).

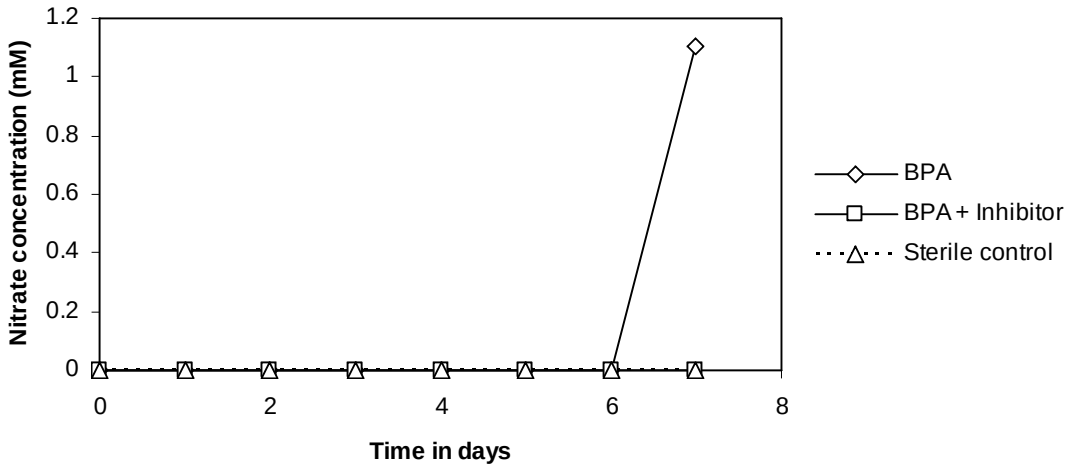


FIG. 4-4c. Nitrate production by activated sludge from a single stage aeration tank with BPA (1 mg/L).

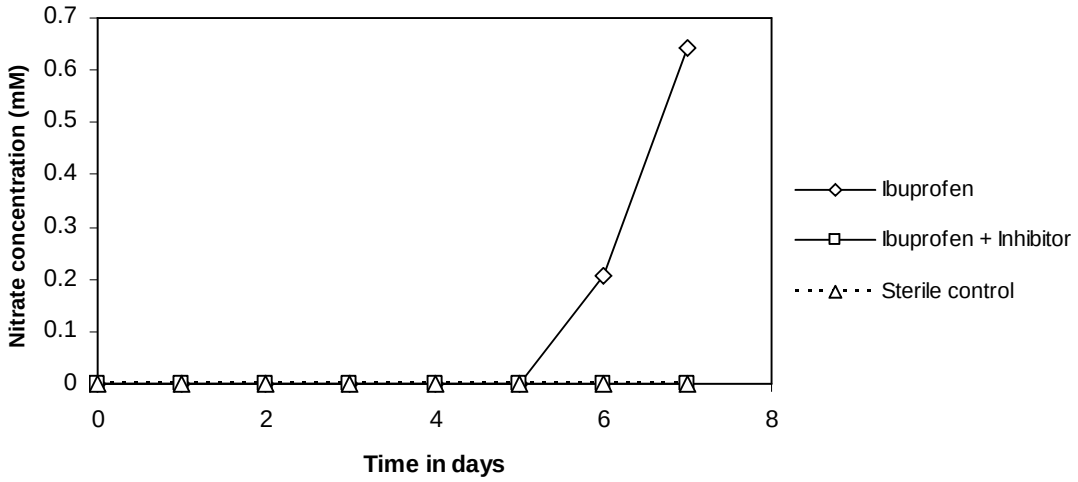


FIG. 4-4d. Nitrate production by activated sludge from a single stage aeration tank with ibuprofen (1 mg/L).

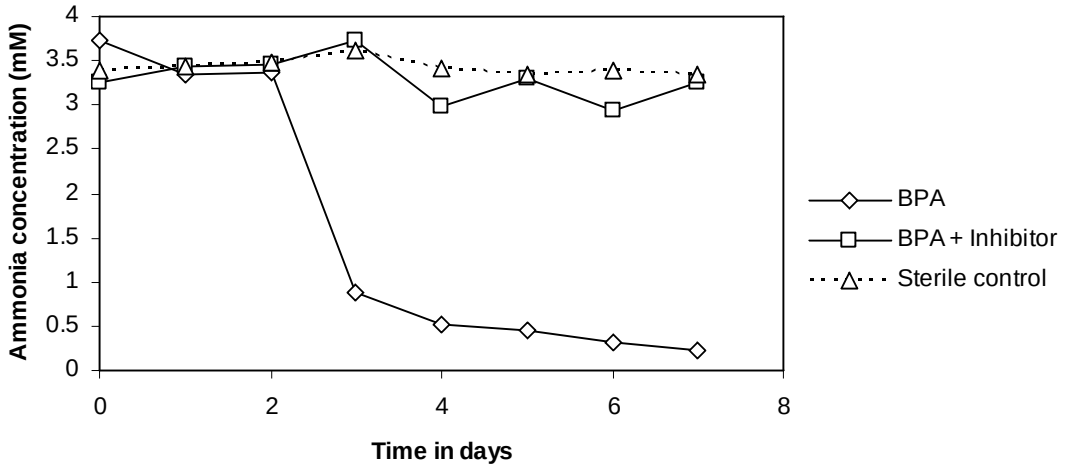


FIG. 4-4e. Ammonia oxidation by activated sludge from a single stage aeration tank with BPA (1 mg/L).

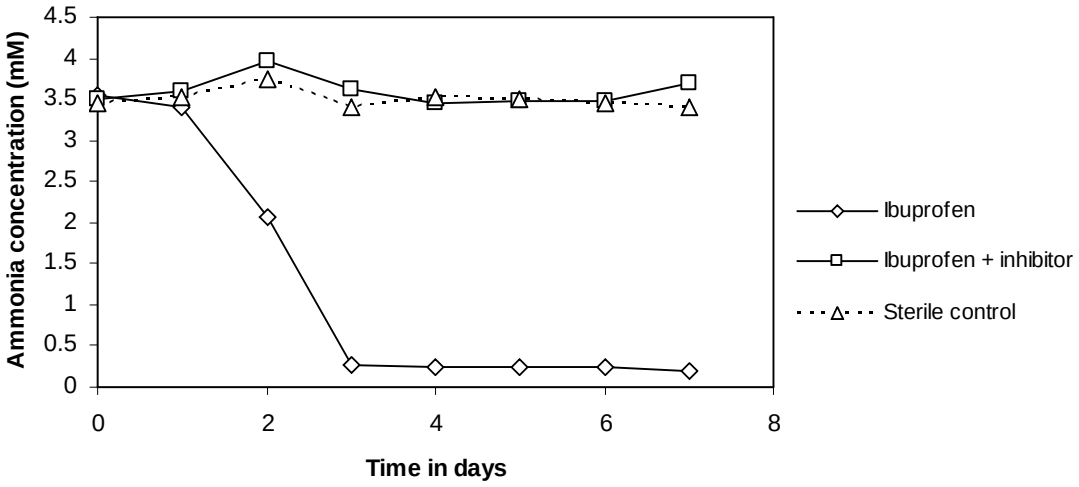


FIG. 4-4f. Ammonia oxidation by activated sludge from a single stage aeration tank with ibuprofen (1 mg/L).

4.2.2 Biodegradation by activated sludge from dual stage treatment process (nitrifying tank). Degradation tests were conducted on BPA of 1 mg/L and ibuprofen of 0.5 mg/L with two stage activated sludge. In both the cases BPA and ibuprofen were completely removed from the system (Fig. 4-5a and b).

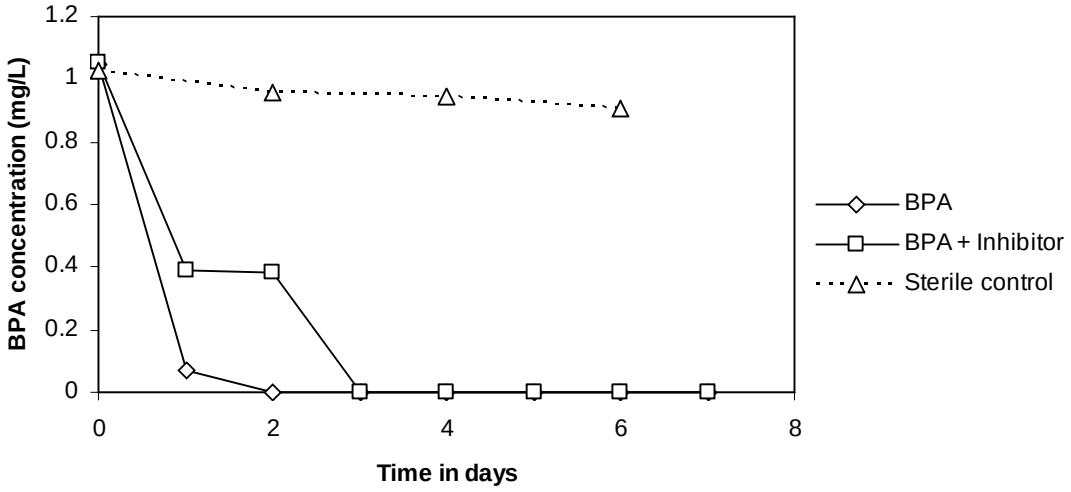


FIG. 4-5a. BPA (1mg/L) degradation by two stage activated sludge.

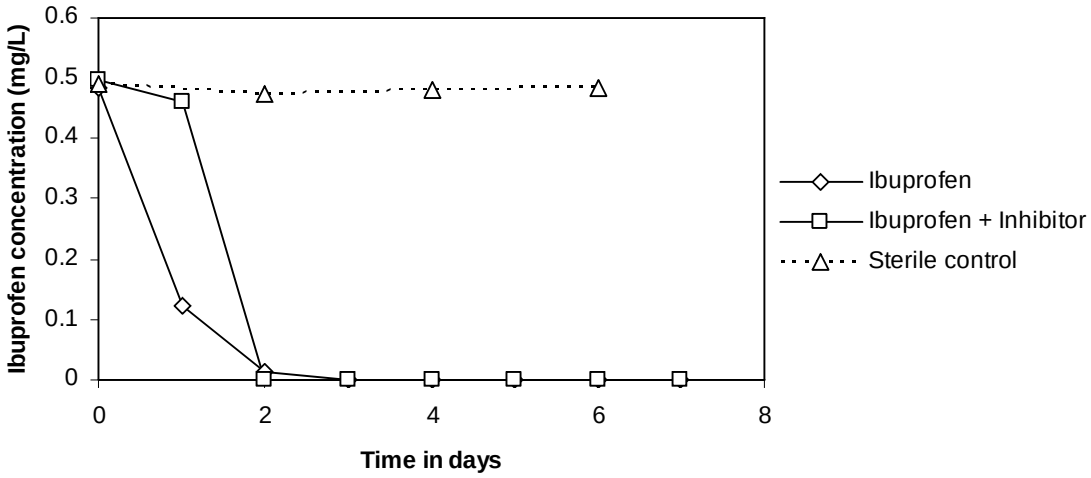


FIG. 4-5b. Ibuprofen (0.5 mg/L) degradation by two stage activated sludge.

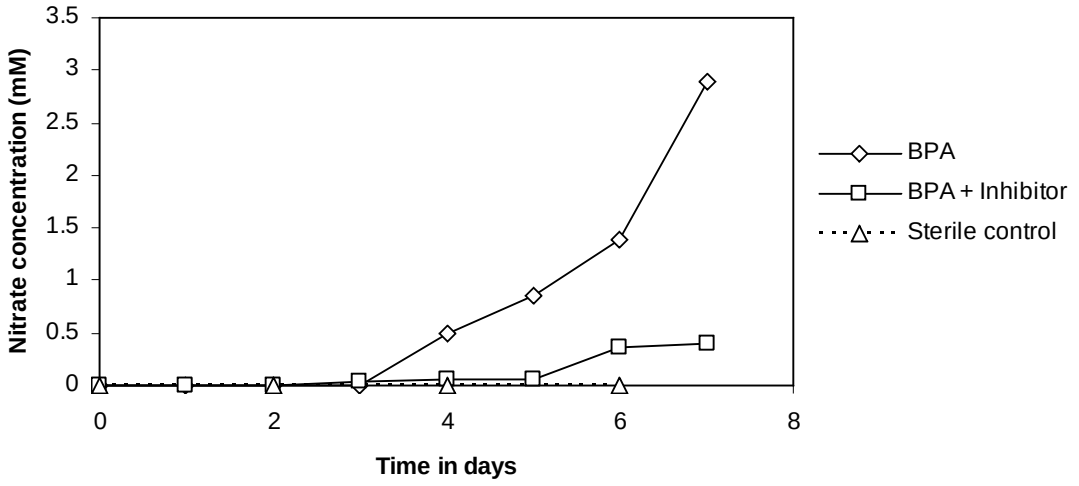


FIG. 4-5c. Nitrate production by two stage activated sludge and BPA of 1 mg/L.

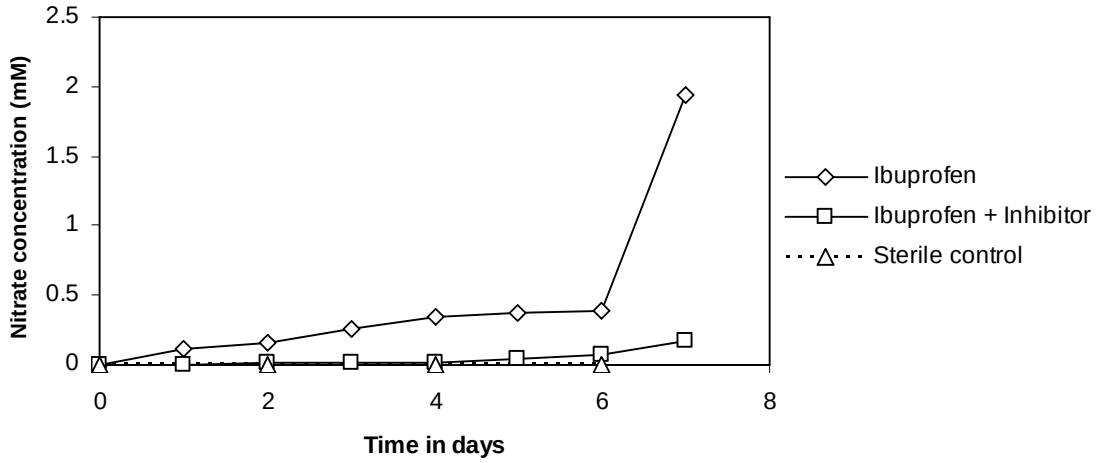


FIG. 4-5d. Nitrate production by two stage activated sludge and ibuprofen of 0.5 mg/L.

The nitrate production and ammonia oxidation is given by Fig. 4-5c and d and Fig. 4-5e and f respectively.

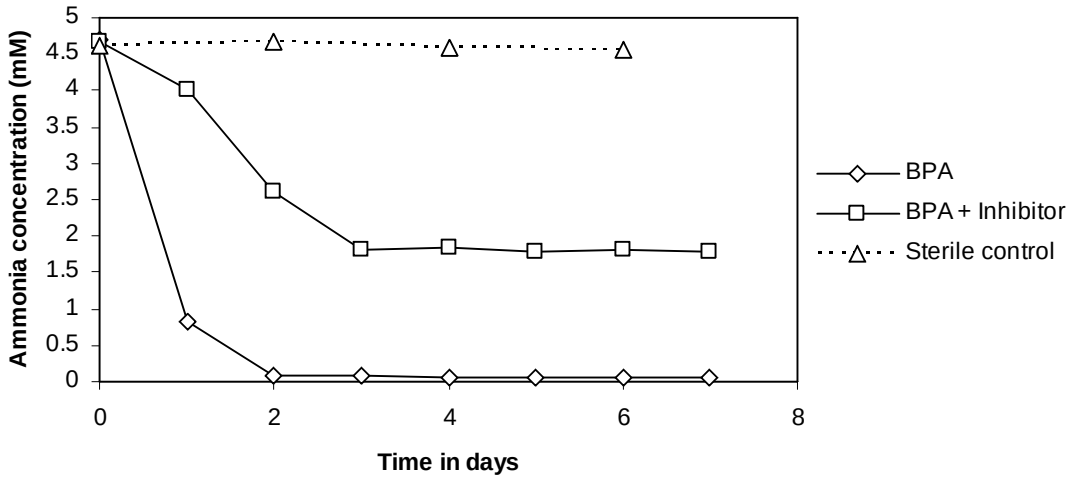


FIG. 4-5e. Ammonia oxidation by two stage activated sludge and BPA of 1.0 mg/L.

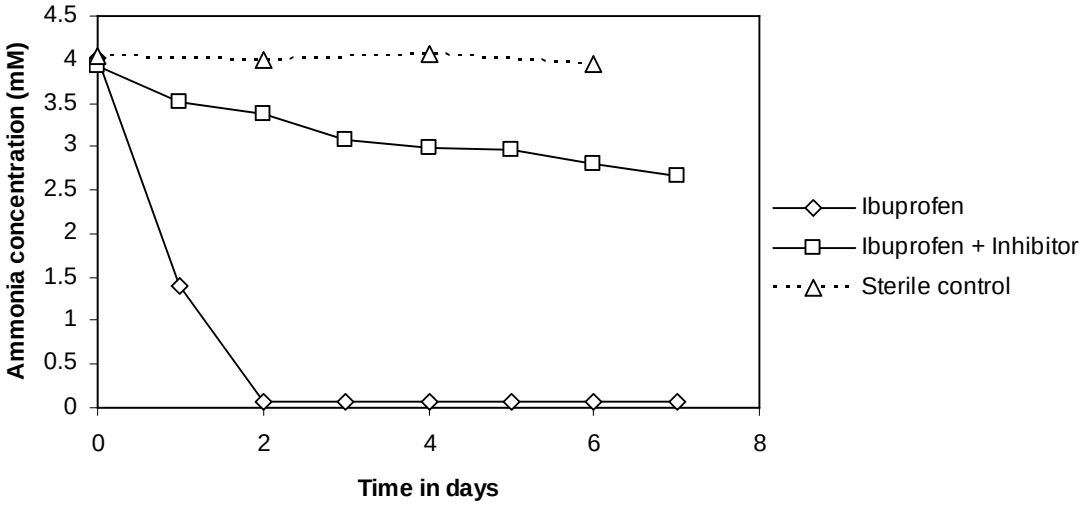


FIG. 4-5f. Ammonia oxidation by two stage activated sludge and ibuprofen of 0.5 mg/L.

Table 10 gives the degradation rates of the experiments conducted with activated sludge on both BPA and ibuprofen. The degradation rates were calculated by considering the initial change in concentration of the compounds over the period

required for the change in concentration. The rate was then normalized by dividing it by the MLVSS of the activated sludge.

TABLE 10. Degradation rates of the experiments conducted with activated sludge on both BPA and ibuprofen.

Experiment	**Initial specific degradation rate (mass BPA/ mass MLVSS x h)**
BPA of concentration 1 mg/L with activated sludge from combined reactor with MLVSS 1000 mg/L. 1. BPA only 2. BPA + Inhibitor	1. 2.7 E -04 2. 2.66 E -05
BPA of concentration 1 mg/L with activated sludge from nitrification tank with MLVSS 1000 mg/L. 1. BPA only 2. BPA + Inhibitor	1. 5.25 E -04 2. 3.16 E -04
Ibuprofen of concentration 0.5 mg/L with activated sludge from combined reactor with MLVSS 1000 mg/L. 1. Ibuprofen only 2. Ibuprofen + Inhibitor	1. 2.6 E -04 2. 2.65 E -04
Ibuprofen of concentration 0.5 mg/L with activated sludge from nitrification tank with MLVSS 1000 mg/L. 1. Ibuprofen only 2. Ibuprofen + Inhibitor	1. 2.37 E -04 2. 2.49 E -04

CHAPTER V
DISCUSSION

Biodegradation is very broadly known as the process by which organic compounds are degraded by living organisms. This naturally occurring process can be utilized for the removal of pollutants from the environment. This study deals with the biodegradation of BPA and ibuprofen with ammonia oxidizing bacterium *N.europaea* and activated sludge from WWTPs.

5.1 BIODEGRADATION OF BPA AND IBUPROFEN BY *N.europaea*

N.europaea is one of the most commonly found AOB in nature [28]. As described in Chapter II, it has been utilized for the degradation of several compounds and pollutants. The activity (non-beneficial mechanism) of the AMO enzyme is known to be mainly responsible for the degradation [25]. Several of the compounds in the substrate range of the enzyme are aromatic in nature [25]. Both BPA and ibuprofen are aromatic compounds and hence are similar to some of the compounds in the substrate range of the AMO enzyme [25].

5.1.1 Degradation of BPA by *N.europaea.* BPA was degraded successfully by *N.europaea* with different ammonia concentrations (Fig. 4-1a and 4-2a) and with a lower cell mass (Appendix I). The oxidation of ammonia and production of nitrite could be observed only in the samples without inhibitor (Fig. 4-1c and d and 4-2c and d). However there was no degradation of BPA observed in the presence of the AMO inhibitor allylthiourea (Fig. 4-1a and 4-2a). This means that allylthiourea successfully inhibited the AMO enzyme. The results also indicate that AMO enzyme was active in the samples without inhibitor and was likely an important step in BPA degradation. This view was further proven by the naphthalene oxidation assay that showed high AMO activity at the start of the experiment and decreased as the degradation of BPA proceeded (Fig. 4-1b and 4-2b).

5.1.2 Effect of ammonia concentration. The ammonia concentration, the presence or absence of ammonia during the reaction and growth of the bacteria impacted the degradation reaction. There was no BPA degradation in the experiment with resting cells. The AMO activity was very low in the absence of ammonia (Appendix I). Contrary to one study reports no loss in ammonia oxidizing activity in the absence of ammonia [51]. From the results of the current study, the presence of ammonia seems to be essential for the degradation reaction to occur as it has a bearing on the AMO activity.

Similarly cells were also grown on two ammonia concentrations of 5 mM and 10 mM. An earlier study had observed higher degradation at 5 mM ammonia concentration [24]. It could be seen that the cells grown on 10 mM had a higher AMO activity of 0.14 compared to the cells grown on 5mM (0.07) based on the naphthalene oxidation tests (in Fig. 4-1b). Corresponding to the higher AMO activity as there was complete removal of BPA within the first 3 ½ hours for the cells grown on 10mM ammonia (Fig. 4-2b). The complete removal took a longer time of 48 hours for 5mM ammonia cells (Fig. 4-2a). The normalized initial specific ammonia oxidation rates for 5mM ammonia and 10mM ammonia was reported to be 5.51×10^{-3} (mass ammonia/mass MLVSS*h) and 1.92×10^{-2} (mass ammonia/mass MLVSS*h) respectively. Similarly the normalized initial specific nitrite production rates were 2.27×10^{-3} (mass nitrite/mass MLVSS*h) and 5.98×10^{-3} (mass nitrite/mass MLVSS*h) for 5mM and 10mM ammonia respectively. From the above rates, the ammonia oxidizing ability of *N.europaea* was seen to be higher for the ammonia concentration of 10 mM. This confirms an earlier finding that reported the loss of ammonia oxidizing activity in AOB under ammonia limiting conditions [51]. The concentration of ammonia thus is an important parameter to be considered in the experiment design.

5.1.3 Degradation of ibuprofen by *N.europaea.* There was no degradation of ibuprofen observed in both the experiments conducted. Even with the higher AMO activity (Fig. 4-3b) in the case of the second experiment, there was no oxidation of ibuprofen (Fig. 4-3a). However there was ammonia oxidation and nitrite production in the absence of the inhibitor indicating the presence of AMO.

The AMO enzyme oxidizes organics by inserting an oxygen atom between the carbon atoms. Due to the difference in the structure of BPA and ibuprofen, a difference in the action of the enzyme on the two compounds is expected. Accordingly, it is probable that AMO is unable to oxidize ibuprofen.

In the case of higher concentration of ibuprofen, the AMO activity was very low (Fig. 4-3b) which could be due to the toxicity of ibuprofen to the bacteria. However no studies have reported the toxicity effects of ibuprofen on bacteria.

5.2 BIODEGRADATION OF BPA AND IBUPROFEN BY ACTIVATED SLUDGE

It is known from previous literature that the BPA removal efficiency can be greater than 90% and the ibuprofen removal greater than 95% in WWTPs [11, 42, 49]. A major portion of the removal of these compounds in WWTPs has been attributed to biodegradation [11, 42, 49].

The activated sludge from WWTPs is known to be the home of many different kinds of bacteria including ammonia oxidizing bacteria [46]. BPA was found to be degraded by the pure culture *N.europaea* while ibuprofen was not. In order to test the degradability of BPA and ibuprofen by the mixed culture, experiments were conducted with activated sludge.

The activated sludge was obtained from two different types of WWTPs. The first experiment was conducted with activated sludge obtained from the local WWTP in College Station. The sludge was collected from a single reactor that was devoted to both BOD and nitrogen removal. The second experiment was conducted with the activated sludge obtained from the Houston WWTP. The Houston WWTP consisted of two stage process, in which one tank was devoted to BOD removal and the other tank to nitrogen removal. The activated sludge for the experiment was obtained from the nitrification tank. Hence it was expected that the AOB population in the activated sludge from the nitrification tank was much higher than the local WWTP.

5.2.1 Biodegradation of BPA and ibuprofen by activated sludge from combined reactor. BPA was degraded by the activated sludge from 1 mg/L to 0.2 mg/L by the 3rd

day and ibuprofen was completely removed by the 2nd day. Hence both the compounds were degraded by the sludge from the combined reactor.

Degradation was observed even in the presence of the inhibitor allylthiourea. Since allylthiourea inhibits AMO or AOB, the degradation in this case could be attributed to other organisms present in the sludge. The production of nitrate started a day or two after ammonia oxidation suggesting the presence of other organisms which could have utilized ammonia. The combined reactor was known to have a lower number of AOB.

5.2.2 Biodegradation of BPA and ibuprofen by two stage activated sludge. BPA and ibuprofen were completely removed by the two stage activated sludge within the first 2-3 days. Hence both the compounds were successfully degraded by the activated sludge.

Once again degradation was observed even with the addition of AMO inhibitor allylthiourea. Since the activated sludge was obtained from the nitrification tank of the WWTP, a higher AOB population in the nitrification tank was expected as compared to that from the combined aeration tank used. Despite the inhibition of activity of AOB by allylthiourea faster degradation of both the compounds was observed. This further strengthens the theory that several other organisms play an important role in the degradation reactions.

From previous literature, BPA removal by bacteria isolated from river water and activated sludge varied between 2- 4 days [20, 22, 23, 32, 43]. The results from the current study were similar with BPA being removed by the 3rd day from activated sludge from the combined reactor and from the 2-3rd day from the two stage reactor. Ibuprofen too was degraded after a span of 2-3 days from a past study by *Sphingomonas* sp. strain [37]. Similar to the previous results ibuprofen removal by the combined reactor was 2 days and 2-3 days by the single reactor.

CHAPTER VI
CONCLUSION AND FUTURE STUDIES

There were two main goals that were achieved by this study. The first was to characterize the degradation of BPA and ibuprofen by a pure culture *N.europaea.* The second was to observe their degradation by activated sludge from two WWTPs. The results and findings of the study are summarized below.

The degradation studies on BPA and ibuprofen by the pure culture *N.europaea* was summarized in Chapter IV. It was found that BPA with a concentration up to 1.6 mg/L was degraded to more than 80% in the experiments on the pure culture. Variations in the experimental setup were introduced to the experimental setup by changing the cell mass, growing the culture on different ammonia concentrations and using resting cells. It was observed that both ammonia and BPA were oxidized by *N.europaea* during the course of the experiment. However, BPA was not be degraded by resting cells probably due to the absence of ammonia. Further, the addition of formate to provide reducing energy seemed unfavorable for the reaction (Appendix I). Also the AMO inhibitor allylthiourea was able to inhibit the oxidation of BPA in all the experimental setups. Due to the inhibition of the AMO enzyme there was no ammonia oxidation or nitrite production observed.

It was observed from the tests conducted on ibuprofen that the compound was not degradable by the pure culture. Experiments were conducted at two concentrations, 1 mg/L and 0.5 mg/L. At the higher concentration, low AMO activities indicated by the naphthalene oxidation assay supported by low ammonia oxidation and nitrite production were observed. In the case of the lower concentration, even though the AMO activity was high there was no degradation of ibuprofen. However this did not interfere with oxidation of ammonia to nitrite.

The pure culture experiments were followed by the experiments with the mixed culture. The first set of experiments were conducted with activated sludge from a combined reactor for both BOD and nitrogen removal. While BPA was degraded to more than 80% within 3 days ibuprofen was completely degraded by the 2nd day by the

activated sludge. The second experiment was conducted with activated sludge from the nitrification tank. Both BPA and ibuprofen were completely degraded by the end of the 2nd day.

BPA degradation by *N.europaea* varied with the experimental conditions. Complete removal by cells grown on 10mM was under 4 hours which was faster than the removal in 2 days accomplished by activated sludge. However complete removal by cells grown on 5mM took a longer time of 30 hours. The reported value of Km for ammonia from previous literature was 1.34mM [24]. In comparison the normalized initial specific ammonia oxidation rate from 5mM ammonia which was lesser than the previous reported. In the case of 10mM ammonia the value of 1.92mM was calculated which is very close to the reported value. The faster degradation by the pure culture could be attributed to the usage of higher cell mass which could be higher than the AOB population in the activated sludge. In the case of ibuprofen there was no degradation achieved by the pure culture while ibuprofen was completely degraded by the activated sludge within 2-3 days.

6.1 FUTURE STUDIES

The pure culture experiments conducted as a part of this study did not include an examination of the kinetic parameters involved in the oxidation of BPA. The main focus of the study was to examine the possibility of BPA biodegradation. During the course of the study, there was no BPA degradation observed by resting cells. The resting cell experiment could be repeated with a higher cell mass and with a lower BPA concentration to confirm the result obtained.

The result obtained from the experiment conducted with sodium formate was unexpected. An experiment could be designed to investigate the role played by an external energy source like sodium formate on the reaction. Some of the other unanswered questions from the study such as the metabolites from the reaction, the reaction pathway and the toxicity of the products to the bacteria require further

investigation. Further, similar degradation experiments could be with *N.europaea* on other organic contaminants that have similar chemical structure as BPA.

Since it was found that ibuprofen could not be degraded by *N.europaea*, similar experiments can be conducted with other AOB or some other non AOB. Further the degradation capability of *N.europaea* with respect to other pharmaceuticals compounds detected in the environment can found out. Since *N.europaea* is an important AOB found in WWTPs, it can be applied in WWTPs for the removal several other important environmental contaminants.

Further BPA and ibuprofen were completely degraded by the organisms present in the activated sludge from both WWTPs. However a faster and more complete removal of both compounds was observed from the nitrification tank. This could be due to the presence of higher number of AOB indicating that they do have role to perform in the degradation. However there was degradation observed even with the addition of the AMO inhibitor in both experiments. No nitrite production was observed in the samples from both the WWTPs as the ammonia was converted to nitrate. This indicates the role played by other organisms in the activated sludge. Hence studies can focus on understanding the role played by other organisms in the degradation occurring in WWTPs even though they are extremely complex.

These studies showed that AOB can cometabolically degrade BPA but not ibuprofen. It is possible to promote a higher AOB population in WWTP to enhance BPA removal. For example, by adjusting operating parameters, such as the sludge retention time and the C/N ratio, might be able to stimulate the growth of AOB and thereby achieve maximum removal of BPA from WWTPs. Consistent with other reports, this study observed complete degradation of ibuprofen by activated sludge, suggesting that ibuprofen-degrading cultures and/or consortia might be ubiquitous in activated sludge systems. Interestingly, only two strains capable of degrading ibuprofen have been isolated [5, 37]. Thus, more studies are needed to better understand the identity and characteristics of ibuprofen-degrading cultures in activated sludge.

Furthermore, studies are needed to examine other potential ibuprofen degradation pathways by various organisms in surface water, sediments, and soils.

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APPENDIX I

1. BPA (1.6 mg/L)

The degradation test was conducted in 1000ml flasks with a final volume of 300ml and optical density of 0.076 or MLVSS of 229 mg/L. The cells were washed with phosphate buffer solution for this experiment only. A concentration of 1.6 mg/L BPA was maintained throughout the degradation test. The setup contained 8 flasks of which 2 flasks contained BPA with the culture. The inhibitor allylthiourea was added to two more flasks and sodium formate to two more. The final set of flasks contained the kill control.

Degradation of BPA from 1.6 mg/L to 0.1 mg/L was observed (Fig. 7-1a) with no degradation in the presence of the inhibitor. However degradation was observed after a period of 20 hours possibly due to the low cell mass used in the experiment. The nitrite and ammonia concentrations are shown in Fig 7-1b and 7-1c. There was no nitrite production or ammonia oxidation observed in the presence of the inhibitor.

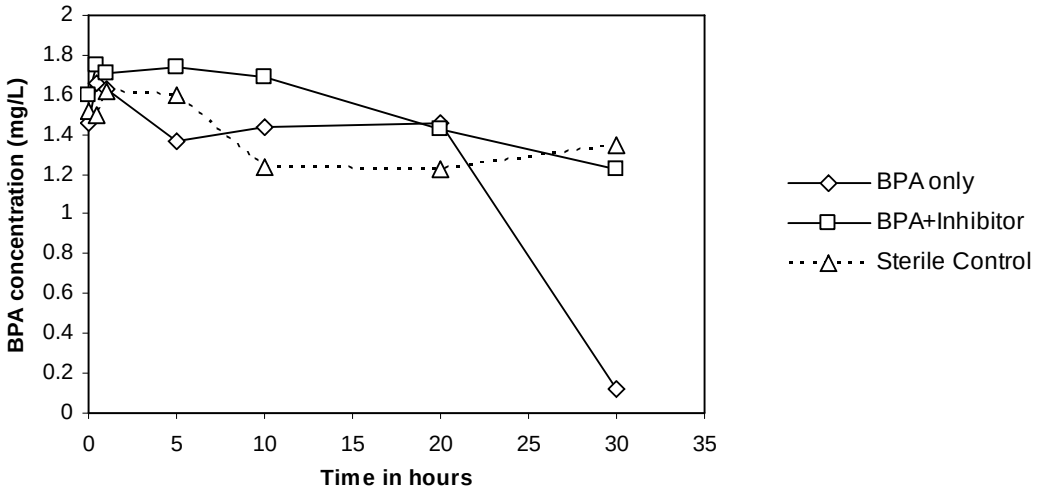

FIG. 7-1a. Degradation of BPA (1.6 mg/L) with and without AMO inhibitor allylthiourea.

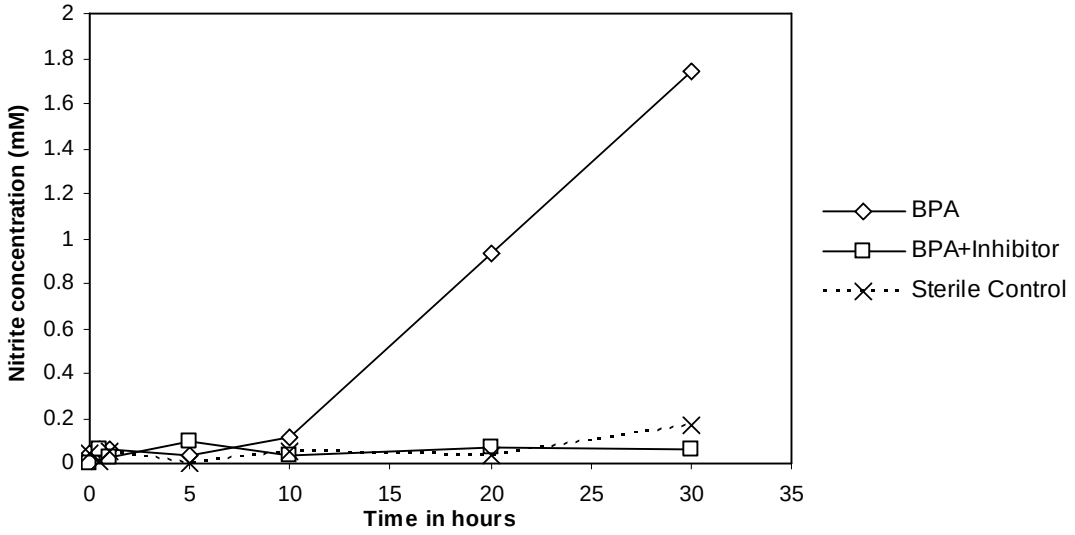

FIG. 7-1b. Nitrite production for BPA (1.6 mg/L) with initial ammonia concentration of 50mM.

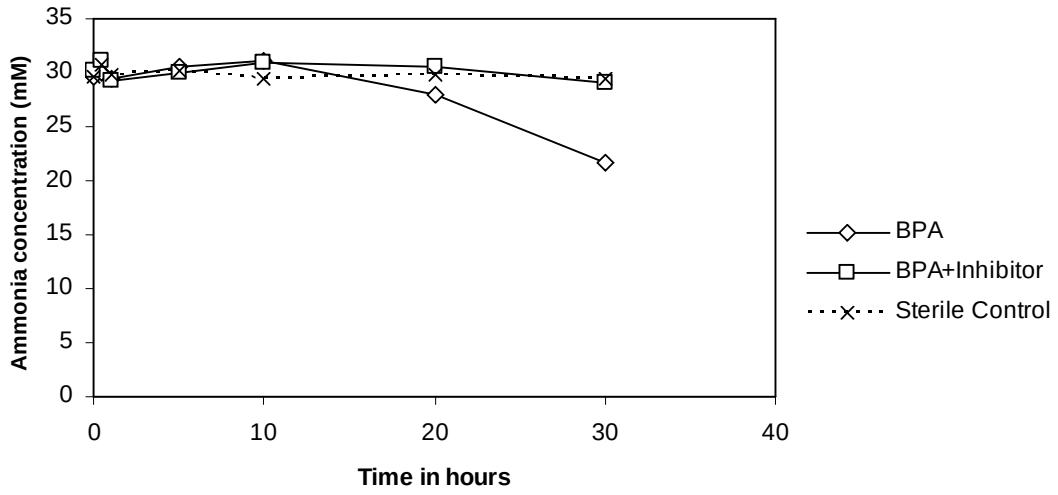

FIG. 7-1c. Ammonia oxidation with BPA (1.6 mg/L) and initial ammonia concentration of 50 mM.

2. Effects of formate on BPA degradation

20mM sodium formate was added to provide reducing energy for the degradation of BPA (1.6 mg/L). There was no degradation observed with addition of formate (Fig. 7-2a) while BPA was degraded from 1.6 mg/L to 0.12 mg/L in the absence of formate. The nitrite production and ammonia oxidation is given by Fig. 7-2b and c.

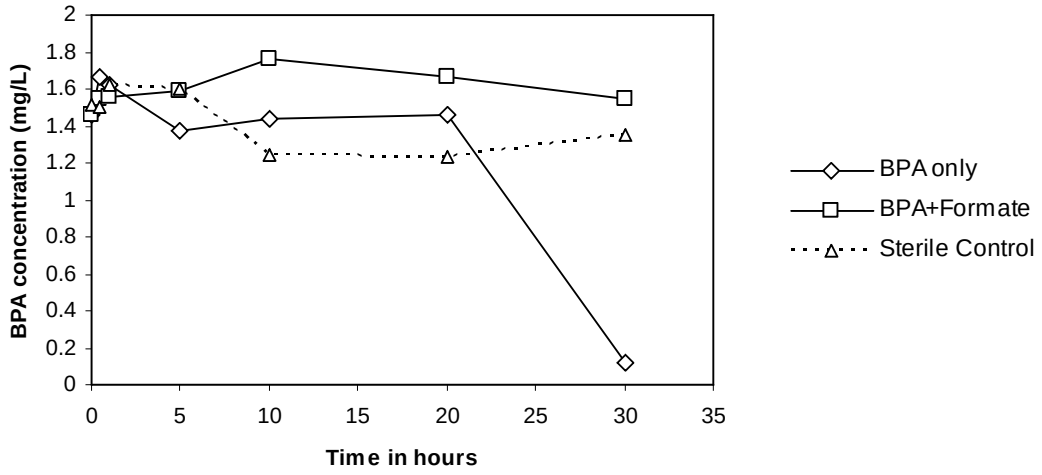

FIG. 7-2a. Degradation of BPA (1.6 mg/L) with and without the addition of 20 mM sodium formate.

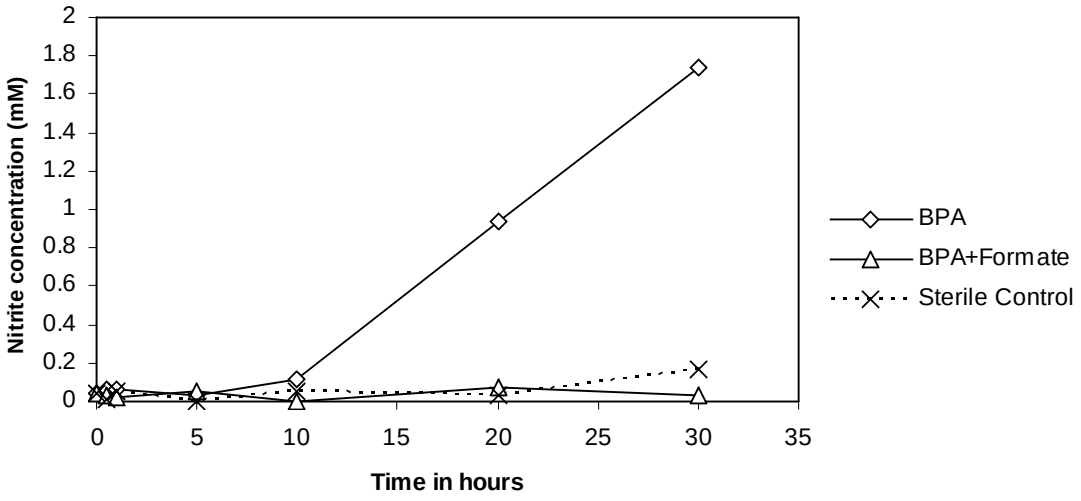

FIG. 7-2b. Nitrite production with and without the addition of 20 mM sodium formate.

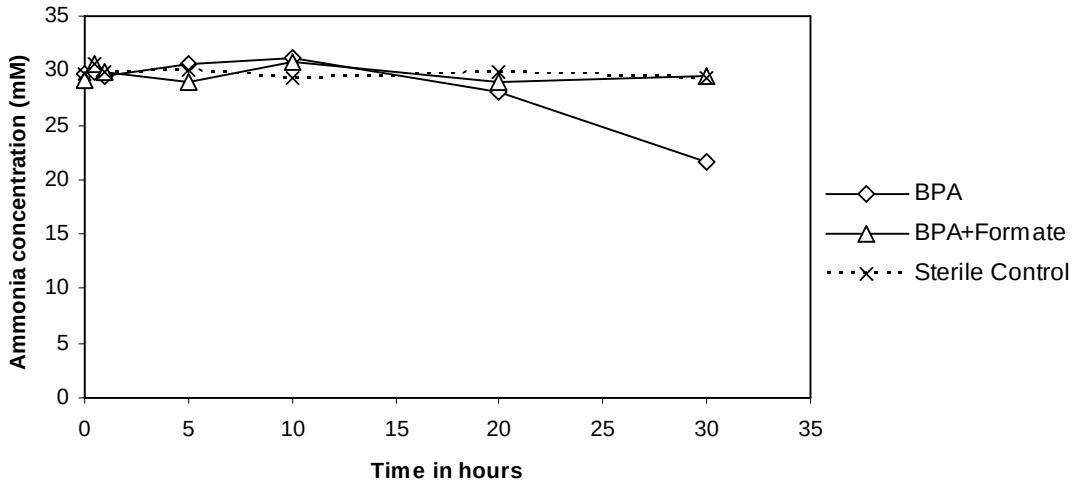

FIG. 7-2c. Ammonia oxidation with and without the addition of 20 mM sodium formate.

3. BPA experiment with resting cells

The resting cells are cells which are first grown in a media containing the primary substrate that the cells utilize as an energy source for their growth. This is done to induce the production of AMO enzyme in the cells [44]. After this stage the cells are starved of

their energy source by transferring them to a media without the primary substrate [44]. The organisms are then forced to use the other available compounds for their growth requirements [44].

The cells for the experiment were grown on 50 mM ammonia and then re-suspended in the media without ammonia. The optical density and MLVSS in a final volume of 300ml was 0.076 and 229 mg/L respectively. The experimental setup excluded the inhibitor allylthiourea.

From the results (Fig. 7-3a) it could be seen that there was no BPA degradation observed. Also the cell activity measured by the naphthalene oxidation assay was low as shown in Fig. 7-3b.

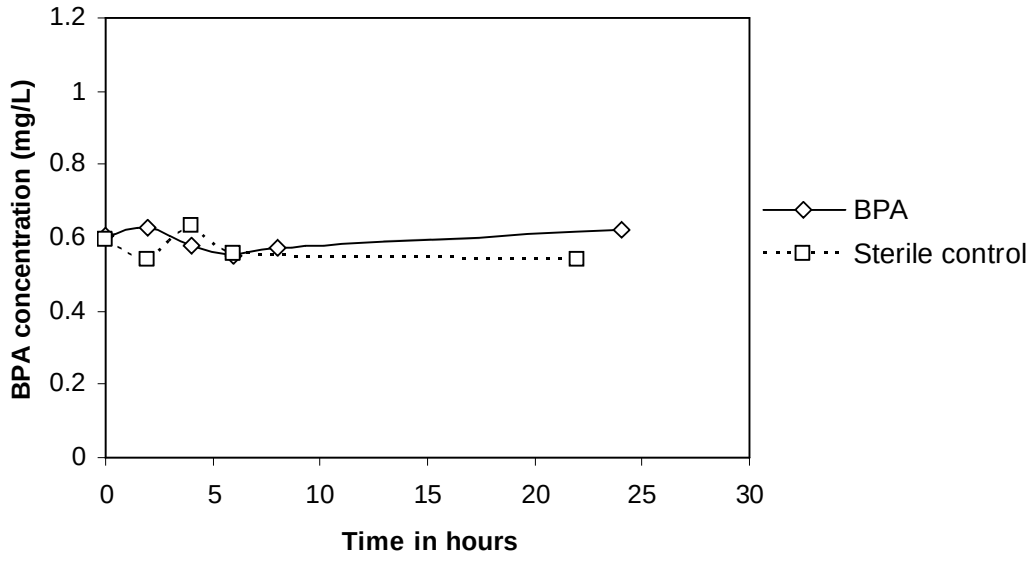

FIG. 7-3a. Degradation of BPA (0.6 mg/L) with resting cells (absence of ammonia).

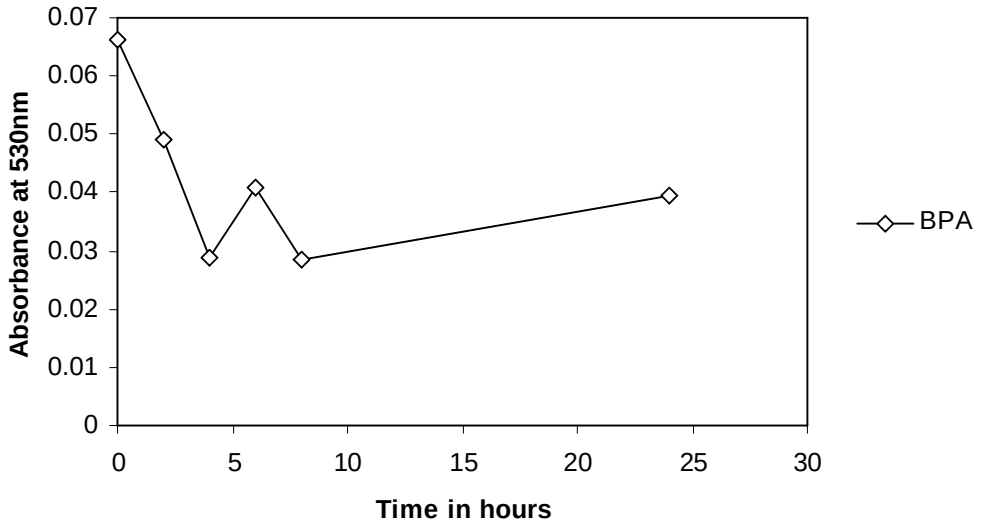

FIG. 7-3b. Naphthalene oxidation assay for resting cells (absence of ammonia).

4. Ibuprofen (0.42 mg/L) degradation experiment

Degradation experiment with ibuprofen of lower concentration was repeated after the experiment with higher concentration in Chapter IV. There was no degradation observed as seen in Fig. 7-4a. Fig. 7-4b shows the decrease in cell activity measured by naphthalene oxidation assay. Similarly there was negligible amount of nitrite production and ammonia oxidation (Fig. 7-4c and d).

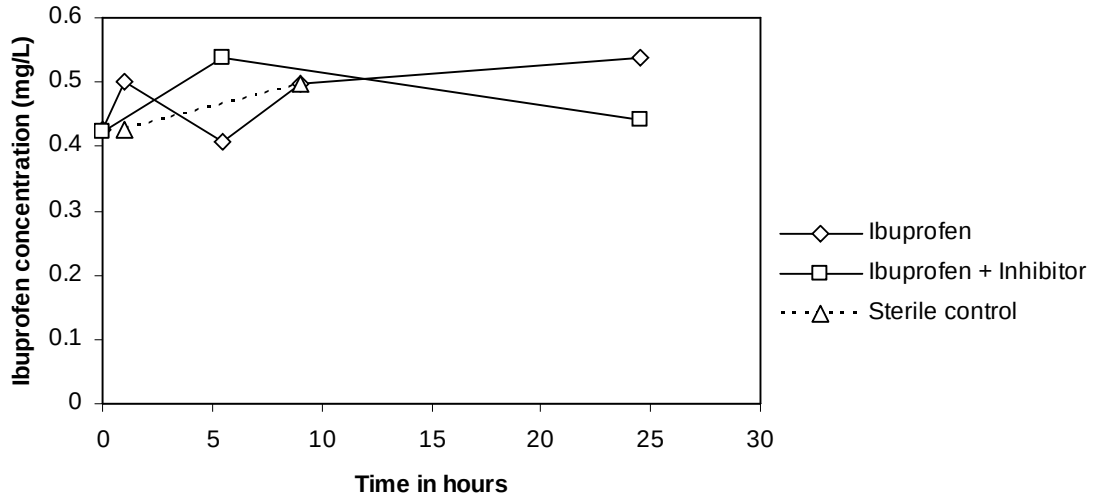

FIG. 7-4a. Degradation of ibuprofen of 0.42 mg/L.

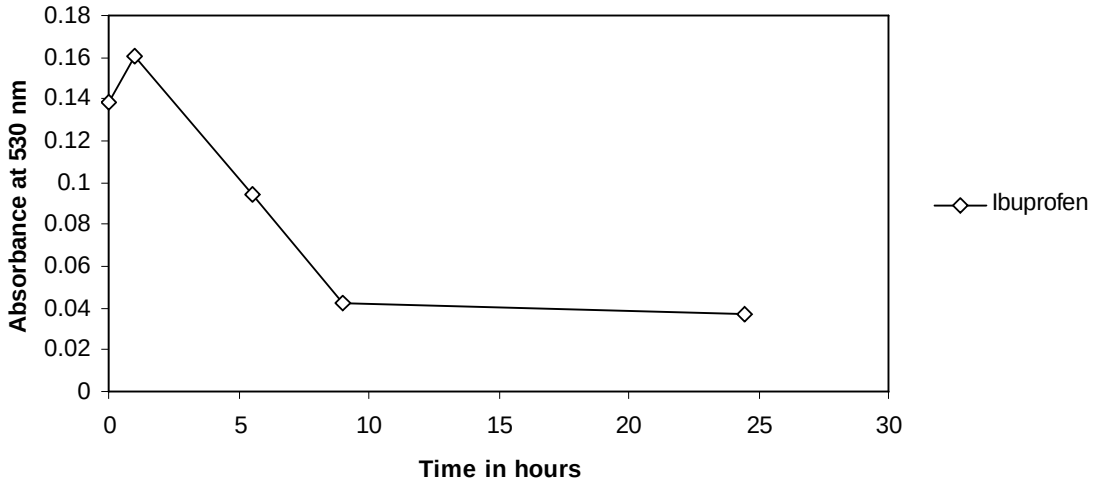

FIG. 7-4b. Naphthalene oxidation assay for ibuprofen of 0.42 mg/L with an initial ammonia concentration 10mM.

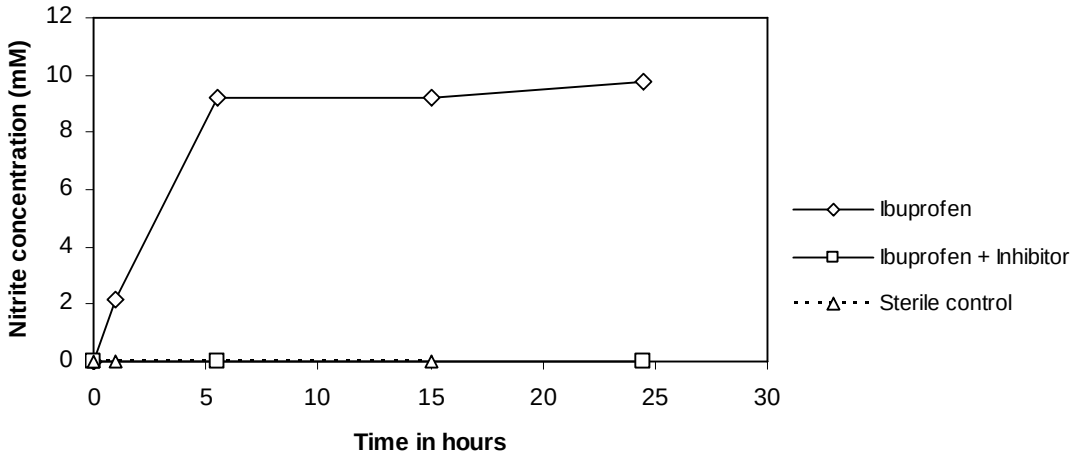

FIG. 7-4c. Nitrite production with initial ibuprofen concentration of 0.42 mg/L , with cells grown on 10 mM ammonia.

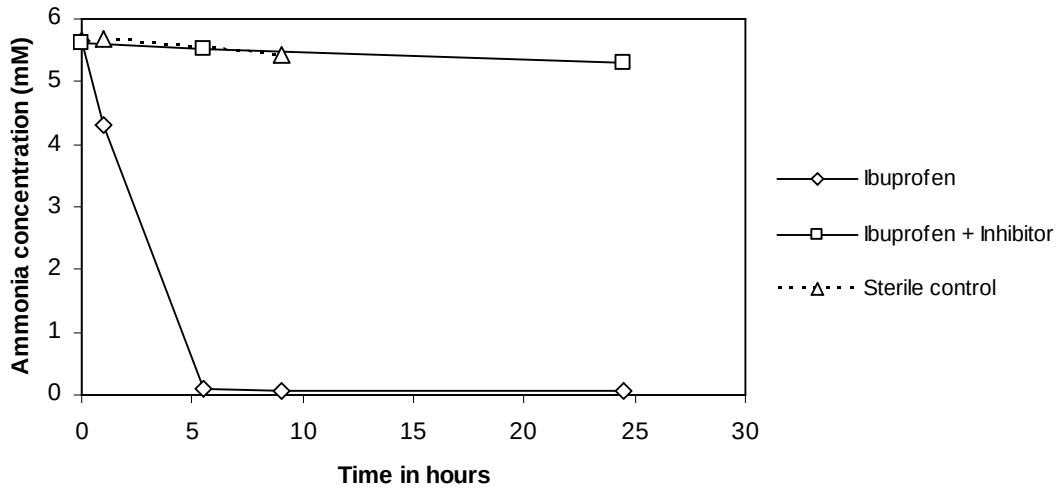

FIG. 7-4d. Ammonia oxidation by *N.europaea* grown on 10mM ammonia with initial ibuprofen concentration of 0.42 mg/L .

VITA

Nethra T. Subramanya graduated from Texas A&M University with a Master of Science degree in civil engineering with her specialization being in environmental engineering. Before joining Texas A&M University in the Fall of 2004, she gained her Bachelors' degree from Visvesvaraya Technological University, India. Her primary research interests include treatment of endocrine disrupters and pharmaceuticals in water and wastewater. Her Masters' research focused on degrading BPA, an endocrine disruptor and ibuprofen, a pharmaceutical using biological method.

Ms. Nethra wishes to pursue her career in the aforesaid areas either through the academia or through industry experience. Her address is 10500 Fountain Lake Dr., Apt.1425, Stafford, TX-77477. She can also be reached at her e-mail address, nethra.subramanya@gmail.com.